SPECIFICATIONS FOR THE CONDUCT OF STUDIES TO EVALUATE THE REPRODUCTIVE AND DEVELOPMENTAL TOXICITY OF CHEMICAL, BIOLOGICAL AND PHYSICAL AGENTS IN LABORATORY ANIMALS FOR THE NATIONAL TOXICOLOGY PROGRAM (NTP)

May 10, 2011

For test-article or study-specific questions
in regard to these specifications,
contact the Contract Officer’s Technical Representative

TABLE OF CONTENTS

I. PERSONNEL

The Principal Investigator, Discipline Leaders for Toxicology, Chemistry, Pathology, Laboratory Animal Management, Health and Safety, Quality Assurance, as well as Study Directors, shall be employees of the testing laboratory (e.g., not consultants or subcontractors). The daily interaction and constant coordination of efforts needed amongst these discipline areas throughout the in-life portion of the studies makes it critical that they be physically and organizationally together.

A. KEY PERSONNEL

Principal Investigator - The Principal Investigator, as key personnel, shall be considered essential to the contract and shall be responsible for the daily management of the contract, point of contact for communication with the NTP Contract Officer's Technical Representative (COTR, also referred to as Project Officer or PO), and in the communication between the NTP personnel and contract personnel. He/she shall be available to meet with NTP personnel during site visits. Site visits by the NTP COTR involving NTP discipline leaders, and quality assurance audits of studies in progress, are routinely conducted at laboratories with ongoing studies. The Principal Investigator must be knowledgeable and up to date in all aspects of the Program/contract, including the status of toxicity studies, record keeping, reporting, and detailed analysis of cost for any or all functions which make up the total operation. General qualifications expected of a Principal Investigator include:

- A Doctorate, or the equivalent relevant experience in a key scientific discipline for toxicology testing (e.g., toxicology, physiology, pathology, veterinary medicine, biochemistry, developmental biology)
- Significant experience in managing large-scale rodent toxicology programs requiring coordination of all disciplines contributing to reproductive and developmental toxicology study programs.
- Demonstrated capability for effective communication, written and oral.

B. DISCIPLINE LEADERS

Individual(s) assigned to the following discipline roles are considered to be "critical staff" and must be capable of meeting the indicated requirements. The Discipline Leaders (DLs) are responsible for establishing scientific guidelines and procedures, training and supervision of professional and technical staff, and evaluation of results and performance within their discipline area relative to NTP requirements.

1. Discipline Leader for Reproductive and Developmental Toxicology
   A Reproductive and Developmental Toxicologist shall be available to supervise and/or work with each Study Director as well as professional and technical toxicology staff. Experience in studying the mechanisms of action of toxic agents is desirable since this individual, in addition to contributing to the implementation of basic experimental designs, must be familiar with any supplementary protocols to be included in study of a particular agent. General qualifications of a DL for Reproductive and Developmental Toxicology include:

   - A Ph.D. or an equivalent in the Biological Sciences with relevant formal course work in disciplines such as toxicology, embryology/developmental biology, anatomy, neurotoxicology, endocrinology, pharmacology, physiology, biochemistry, and related areas. A record of publications in peer reviewed journals, book chapters and/or demonstrated experience in preparation of relevant laboratory study reports. Board certification (American Board of Toxicology) is desirable.

   - Experience in the conduct of developmental toxicity studies as well as multi-generational reproductive toxicity studies in laboratory animals.

- Experience in the management of professional and technical toxicology staff.

2. Discipline Leader for General Toxicology
   A General Toxicologist shall be available to supervise and/or work with each Study Director as well as professional and technical toxicology staff. Experience in studying the mechanisms of action of toxic agents is desirable since this individual, in addition to contributing to the implementation of basic experimental designs, must be familiar with any supplementary protocols to be included in study of a particular agent. General qualifications of a DL for General Toxicology include:

   - A Ph.D. or an equivalent in the Biological Sciences with relevant formal course work in disciplines such as toxicology, pharmacology, physiology, biochemistry, and related areas. A record of publications in peer reviewed journals, book chapters and/or demonstrated experience in the preparation of relevant laboratory study reports. Board certification (American Board of Toxicology) is desirable.

   - Experience in the conduct of a variety of toxicity studies including short and long-term toxicology studies and in the management of professional and technical toxicology staff.

   - Experience with in vivo evaluation of toxicants in rodents of various ages (e.g., whole animal testing, treatment response) preferably in toxicology studies of both short and long-term duration.

3. Discipline Leader for Chemistry
   A Chemist shall be available for supervision of, and/or work with, personnel performing chemical analyses, dose preparations, and dose analyses. General qualifications of a DL for Chemistry include:

   - Ph.D. in chemistry with two years relevant experience or M.S. in chemistry with five years relevant experience.

   - Experience in chemical analyses for purity and identity as well as dose mixing and dose analysis relative to those required in this Specifications plus experience in the use of analytical instrumentation including gas-liquid and high performance liquid chromatography, spectroscopy, instrumental and wet chemistry analyses.

4. Discipline Leader for Pathology
   A veterinary or medical Pathologist with experience in laboratory animal rodent pathology shall be responsible for oversight of professional and technical pathology staff and all pathology procedures, histopathologic evaluations, and reporting. General qualifications of a Pathologist include:

   - Formal training in a medical specialty, e.g., veterinary medicine or medicine.

   - Experience in rodent pathology; particularly reproductive, developmental and toxicologic pathology.

   - Experience in management/supervision of pathology projects and/or staff.

   - Post-doctoral training and/or Board Certification in pathology.

5. Discipline Leader for Laboratory Animal Management
   A veterinarian, with experience in laboratory animal medicine, must closely monitor the health and maintenance of experimental animals, provide oversight for the vivarium, and interact

with the Principal Investigator and the NTP personnel concerning all phases of the animal experiments. General qualifications of the DL for Laboratory Animal Management include:

- Graduate of Veterinary College recognized by the American Veterinary Medical Association.

- Diplomate of the American College of Laboratory Animal Medicine (preferred) or eligible by experience and education to take the examination.

- Previous experience in managing large colonies of laboratory animals, in particular, rodents in a toxicology setting.

- Previous experience that required multidisciplinary interactions such as toxicology studies.

- Previous experience in laboratory animal management involving the conduct of studies using breeding, pregnant and lactating animals.

6. Health and Safety Officer

A qualified Health and Safety Officer shall be designated to monitor worker health and safety conditions during all phases of the work. In his/her role as Health and Safety Officer, he/she shall be responsible to someone other than the Principal Investigator (PI) and the PI's subordinates, and shall have the authority to bring unsafe conditions to the attention of higher management. The Health and Safety Officer may have other responsibilities within the Testing Laboratory's organization; however, the amount of time devoted explicitly to health and safety is to be commensurate with the scale of the Testing Laboratory's operations. General qualifications of the Health and Safety Officer include:

- Bachelor's degree (at a minimum) majoring in industrial hygiene, chemistry, biology, safety engineering or a closely related science or engineering field.

- At least two years experience in occupational health and safety, along with completion of courses in general occupational health and hazard control indicating the acquisition of successively greater levels of knowledge regarding industrial hygiene. This experience shall have taken place within the last 4 years. (A Master's degree in industrial hygiene, safety engineering or a Bachelor's degree in industrial hygiene, safety engineering, with one year of experience, is an acceptable substitute for this experience.)

- Training shall have been completed within the last eighteen months, and will be refreshed with additional training at an interval not exceeding eighteen months.

- Recent experience in working with specific requirements of local, state, and federal statutes relating to occupational health and safety, environmental protection, and chemical monitoring.

- Demonstrated ability to deal effectively with the scientific and managerial staffs in responsibly implementing the health and safety program (including the identification of problem areas and the execution of corrective actions as required).

7. Quality Assurance Unit Officer

This individual shall be responsible for monitoring each study to assure management that the facilities, equipment, personnel, methods, practices, records, and controls are in conformance with the regulations in Part 58, "Good Laboratory Practices for Non-clinical Laboratory Studies (Federal Register, Friday December 22, 1978, Part II and any later interpretations published by the FDA). The Quality Assurance Unit Officer shall have

management support and organizational independence from the personnel engaged in the direction and conduct of the study. General qualifications for a Quality Assurance Officer include:

- A minimum of a Bachelor's degree, majoring in a biological science or chemistry.
- Appropriate scientific experience to ensure understanding of the tasks or reports being inspected or audited in toxicology, chemistry, and histopathology.
- The QAU Officer should have supervisory experience when the unit consists of more than one person.

C. OTHER CRITICAL STAFF

1. Study Directors

   Individual(s) assigned as the GLP Study Director must have formal training in and experience with conducting studies according to regulations defined in Part 58, "Good Laboratory Practices for Non-clinical Laboratory Studies (Federal Register, Friday, December 22, 1978, Part 11 (and subsequent updates/interpretations published by the FDA). The Study Director is expected to have day-to-day interactions with technical staff, observe key in-life/necropsy phases, and must be available to observe all toxicological animal room activities. The Study Director must be capable of meeting the following requirements:

   - A Ph.D. or an equivalent in the Biological Sciences with relevant formal course work (or equivalent) in disciplines such as toxicology, pharmacology, developmental biology, endocrinology, physiology, biochemistry, and related areas. Board certification (American Board of Toxicology) is desirable.
   - Experience with in vivo evaluation of toxicants in studies with laboratory animals of all ages including reproductive and developmental toxicology studies (e.g. rodent reproductive, developmental and/or multigenerational studies) by various routes of exposure. Additional experience with general toxicity studies is desirable.
   - Demonstrated capability for effective communication, especially written.

2. Pathologists

   Veterinary or medical Pathologists with experience in laboratory animal rodent pathology are responsible for the necropsy/histopathologic evaluation and reporting of a study. They must be available to readily respond to necropsy, histology, and other related issues. General qualifications of a Pathologist include:

   - Formal training in a medical specialty, e.g., veterinary medicine or medicine.
   - Experience in rodent pathology including gross and microscopic pathology of the reproductive system, and toxicologic pathology.
   - Post-doctoral training and/or Board certification (American College of Veterinary Pathologists).

3. Breeding Specialist

   Breeding Specialist with experience in the breeding of laboratory animals, particularly rodents such as rats and mice. General qualifications include:

   - Knowledge of reproductive biology gained through education and experience.

- Understanding of mating and maternal behavior, as it applies to the production of offspring for research purposes including toxicology studies.

4. Data Management Coordinator
   Individual responsible for data management activities involving data collection, management, reporting, and archiving.

   - Individual shall have a documented record of experience and training in this area, specifically as it applies to reproductive and developmental toxicity studies.

D. SUPPORT STAFF

1. Dose Formulation and Analysis Staff
   A dose formulation supervisor with experience related to the testing program. Appropriately trained technical staff to perform dose formulation, and bulk test article and dose formulation analysis.

2. Animal Care/Toxicology Staff
   Animal care/toxicology technicians with training and experience in the administration of test articles via the required routes of exposure (including direct dosing of neonates), in providing appropriate animal care, in performing standard reproductive assessments (including observations and activities related to littering parameters, sexing neonates and measurement of ano-genital distance), assessment of development markers of sexual maturation (i.e. vaginal opening and preputial separation), performing neurobehavior assessments, sperm motility and vaginal cytology evaluations, and in evaluating clinical signs of toxicity in pregnant, neonatal and adult animals.

3. Necropsy and Histology Staff
   Prosectors trained and experienced in the anatomy and dissection of rodents (including detailed dissection of reproductive tissues), experienced in the conduct of fetal examinations (external, visceral and skeletal), able to recognize abnormal anatomic organs of neonatal and adult animals and to describe gross abnormalities, including those of involving the reproductive tract. ASCP (HT) or ASCP (MT) registered technologist to supervise the histology operations. Appropriately trained histology technicians.

4. Clinical Pathology Staff
   Registered technologist to supervise clinical laboratory studies. Appropriately trained personnel with experience conducting clinical laboratory tests including hormone analyses.

5. Quality Assurance Staff
   Appropriately trained staff responsible for conducting audits and inspections of study activities.

6. Data Management Staff
   Individual(s) responsible for data management activities involving data collection, management, reporting, and archiving aspects.

BLANK PAGE

II. FACILITY

A. FLOOR PLAN

Laboratories conducting studies for the NTP shall provide floor plans showing locations where all study activities are conducted.

1. Animal Facility
   Floor plan indicating quarantine rooms, animal rooms, showers/change areas and rest rooms; storage areas for feed, bedding and general storage; cage and rack washers; emergency power source and those areas in which emergency power operates. This floor plan is to indicate traffic flow for personnel, animals, test article, feed/bedding, supplies, and equipment through the facility.

   A separate floor plan of the animal facility is to indicate room airflow directionality and indicate the location of all safety equipment, such as eyewash stations, safety showers, fire control equipment, etc.

   A floor plan indicating ventilation equipment and ductwork, including interior and exterior exhausts, shall be supplied. A floor plan for the roof(s) shall indicate the location of each of the building(s) general air intakes and exhausts and the location of the exhaust for each hood or vented enclosure.

2. Chemistry
   Indicate storage areas for bulk chemical and dose formulations, bulk chemical and dose formulation analysis, dose formulation, and supporting equipment and exhaust hoods.

3. Clinical pathology
   Indicate laboratory space and location where specimens are collected and analyzed.

4. Fetal examinations
   Indicate laboratory areas where fetal examinations and processing take place.

5. Pathology
   Indicate areas for necropsy, histology, pathology, and storage.

6. Other areas
   Indicate QA offices and filing space; data archives; waste storage; and special study facilities.

B. EMERGENCY FACILITY SUPPORT

Facilities shall have a tested back-up power source with automatic changeover equipment that is sufficient to preserve the integrity of the testing experiment. Emergency power must handle those areas critical to the study such as animal rooms, HVAC, storage freezers/refrigerators, waste storage, autotechnicons, etc. Essential mechanical equipment must be guarded or alarmed. Provisions for prompt maintenance response must be provided.

C. ANIMAL FACILITIES

All facilities for the Testing Program must be approved by the NTP and will be evaluated with respect to criteria outlined in the "Guidelines for Carcinogen Bioassay in Small Rodents" (DHHS Publication No. (NIH) 76-801), "Long Term Holding of Laboratory Rodents" (ILAR News, XIX, #4, 1976), the "Guide for the Care and Use of Laboratory Animals for Research Involving Chemical Carcinogens" (DHHS Publication No. (NIH) 76-900), the "Guide for the Care and Use of Laboratory Animals NRC, 2010), and any other additions and exceptions thereof. Research is to

be conducted in accordance with the Public Health Services Policy on Humane Care and Use of Laboratory Animals, Office of Laboratory Animal Welfare (OLAW). Accreditation of toxicology research and testing facilities by an external peer review organization such as AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care International) or CCAC (Canadian Council on Animal Care) is required. It is the responsibility of the testing laboratory to establish policy and procedures to address entry of approved staff and visitors into the animal facility. Entry shall be prohibited to those individuals that have been in another animal facility within the last 48 hours regardless of disease status of that facility. The animal facilities shall be designed and managed to prevent contamination of animals with pathogenic organisms and to prevent contamination of personnel and the environment with test articles and also to prevent cross-contamination of animals with other test articles. A two-corridor system with intervening animal rooms is the preferred way to fulfill this requirement, given that:

1. All materials coming in contact with animals are sanitized to a clean state suitable for introduction to the supply (clean) corridor and the animal rooms.

2. Following use, these materials are removed from animal rooms by a return (dirty) corridor for disposal, destruction, or reprocessing.

3. The air pressure is adjusted so that the animal rooms are positive to the return corridor and negative to the "clean" one.

4. In cases where animals must be moved from one room to another, such as in multigeneration studies or conduct of neurobehavior tests, precautions must be followed so that the barrier system is not compromised.

D. SUPPORTING FACILITIES

1. Animal Facility

a. Facilities for sanitization of cages, racks, water bottles, and feeders must be available and properly located in relation to the study rooms.

b. Clean, well-ventilated, vermin-free storage space must be provided for clean supplies and equipment, cage filters, and feed/bedding awaiting use.

c. Ability to autoclave feed and bedding is desirable.

2. Pathology

a. The necropsy facility must be equipped with adequate working surfaces, dissection boards, running water with drains, dissection microscopes, perfusion equipment, adequate lighting, ventilation, and exhaust hoods. Necropsy and microscopic photography capabilities are required. The location of pathologists' offices shall allow them to be readily available to address necropsy issues and attend necropsies.

b. Refrigeration shall be available for holding dead animals until necropsy. Dead animals shall not be frozen prior to necropsy.

c. The histology laboratory should be separated from the necropsy area and equipped with automatic tissue processor(s), microtomes, embedding and staining equipment, and with supplies and appropriate ventilation adequate for the expected volume.

d. Acceptable storage space must be available for storage of residual archival and histologic materials that will be retained by the Testing Laboratory prior to shipment to the NTP-designated recipient. These areas must have secured, limited access.

3. Chemistry and Hazardous Waste Storage

    1. The contractor must have the analytical instrumentation required for dose analysis and chemical purity checks. The specific types of equipment required will depend on the test article(s), but might include gas and/or liquid chromatographs, infrared spectrophotometers, UV-visible spectrophotometers, and other similar instruments and apparatus.

    2. Secured, controlled access bulk storage facilities must be available for retention of the test article at 5º C ± 3º C or at ambient temperature as specified by the NTP. Also, cold storage at –20º C ± 5º C must be available for analytical reference standards.

    3. Suitable V-blenders with intensifier bars must be used for diet mixing.

    4. Designated areas shall be provided and described for hazardous waste storage prior to disposal.

4. Data and Quality Assurance Archives

    A secured, limited access area shall be provided for maintenance of all study records. In addition, Quality Assurance records must also be stored in a secured, limited access area.

BLANK PAGE

III. HEALTH AND SAFETY

A. ADMINISTRATIVE CONTROLS

1. Regulations and Guidelines

The NTP and/or its representatives may inspect, photograph, sample and monitor the laboratory and associated facilities used for its studies at any time to ensure that NTP minimum requirements and applicable regulations and guidelines (described below) are being followed. Any deviations to these requirements shall be approved by the NTP.

All work shall conform to applicable local, state, and federal statutes including the following federal regulations and updates:

OSHA:
- Standards for General Industry, 29 CFR 1910
- Hazard Communication, 29 CFR 1910.1200
- Respiratory Protection, 29 CFR 1910.134
- Occupational Exposure to Hazardous Chemicals in Laboratories, 29 CFR 1910.1450
- Occupational Exposure to Blood-borne Pathogens, 29 CFR 1910.1030
- Formaldehyde, 29 CFR 1910.1048 (applicable to the use of formaldehyde in histology, pathology, and anatomy laboratories)

DOJ:
- Americans for Disability Act, Accessibility, Design Guidelines, 28 CFR, Title III, Part 36.

EPA:
- Clean Air Act, 40 CFR 50-80
- Clean Water Act, 40 CFR 100-140 and 400-470
- Resource Conservation and Recovery Act (RCRA) 40 CFR 240-271
- Comprehensive Environmental Response, Compensation and Liability Act (CERCLA, Superfund, SARA) 40 CFR 300.

DOT:
- General Information, Regulations, and Definitions, 49 CFR 171
- Hazardous Material Table, Special Provisions, Hazardous Materials Communication Requirements and Emergency Response Information Requirements, 49 CFR 172
- Shippers, General Requirements for Shipments and Packaging, 49 CFR 173
- Carriage by Public Highway, 49 CFR 177

NRC:
- Standards for Protection against Radiation, 10 CFR 20
- Notices, Instruction, and Reports to Workers; Inspections, 10 CFR 19
- Recommendations described in the most recent version of the NIH Radiation Safety Guide

DEA:
- Federal Requirements for Controlled Substance, 21 CFR 1300

For contract work involving infectious agents, the Centers for Disease Control Guidelines, Biosafety in Microbiological and Biomedical Laboratories (HHS Publication No. (NIH) 93-8395, 2009) and the NIH Guidelines for Research Involving Recombinant DNA Molecules (66 Federal Register 57970, 2001 and updates) shall be followed.

Where not superseded by this document, the American National Standard for Laboratory Ventilation, Z 9.5, published by the American National Standards Institute (ANSI) shall be followed.

Other consensus standards and publications may include: the current edition of the Threshold Limit Values for Chemical Substances and Physical Agents & Biological Exposure Indices published by the American Conference of Governmental Industrial Hygienists (ACGIH), Criteria Documents for various substances and the Recommended Exposure Limits published by the National Institute for Occupational Safety and Health (NIOSH), and the Workplace Environmental Exposure Levels (WEEL) published by the American Industrial Hygiene Association. Where there may be conflict in the acceptable exposure levels as compared to the OSHA Permissible Exposure Limit, the most stringent standard shall be used for worker's protection.

2. Health and Safety Plan (Chemical Hygiene Plan)

a) Health and Safety Plan Scope

The scope of each Health and Safety Plan shall address the organization's health and safety policies, Occupational Medical Surveillance Program, as well as potential chemical, physical, biological and ergonomic hazards (e.g., acquisition of study materials, storage, and handling through ultimate disposal of contaminated wastes).

No contract laboratory will participate in studies without a Health and Safety Plan that has been approved by the NTP. An updated Plan shall be submitted every 2 years to the NTP for review. In addition, the NTP shall be informed of any updates to the Plan during the course of the contract. If approval of the Plan is not granted at the time of award, the laboratory must submit a revised Plan for review within 30 days of the receipt of award notification. Revisions to the Plan shall be clearly indicated to facilitate reviewer approval.

For all contract laboratories, a Chemical Hygiene Plan as required under the OSHA "Laboratory Standard" may be used in place of a Health and Safety Plan provided it meets or exceeds ALL of the requirements outlined in this document.

b) Health and Safety Plan Content

Written Policies. In addition to the SOPs outlined below, the Health and Safety Plan shall address (but not be limited to):

- health and safety responsibilities, policies and organization
- record keeping and archiving
- initial and periodic employee training
- engineering controls
- personal and environmental monitoring
- medical surveillance and biological monitoring
- respiratory protection program
- personal protective clothing and equipment
- general housekeeping
- eating and smoking policies and areas
- precautionary signs and labels
- chemical and biological storage
- fire protection and prevention
- emergency and evacuation contingencies

- locations (with schematic diagrams) of fire control equipment, and plumbed eyewash stations and emergency showers
- laboratory safety inspection
- waste management and disposal
- other pertinent personnel, operational, and administrative practices, and engineering controls necessary for the containment and safe handling of chemical, physical, biological, and radiological hazards.
- entry and exit to restricted areas
- visitors

3. Standard Operating Procedures

The laboratory shall be required to have Standard Operating Procedures (SOPs) that have been reviewed and approved by NTP for at least the following activities:

- visitors access to test areas
- employee training
- medical surveillance and biological monitoring
- respiratory protection, mask-fit, cleaning/maintenance, and inspection
- eye and face protection
- personal protective clothing and equipment
- general housekeeping practices
- ventilation system maintenance
- storage, receipt, transport, and shipping of study materials
- hazardous material handling (e.g., in analytical chemistry labs)
- dose preparation (if applicable)
- entry and exit from the limited access areas (including traffic patterns of dose prep facility and animal handling and testing room) (if applicable)
- spill clean-up, accident, emergency response and evacuation (including natural disasters) and fires/explosions
- use of radio-labeled material, infectious agents, and/or controlled substances (if applicable)
- waste management and disposal

4. Exposure Evaluation and Control

a) Permissible Exposure Limits/OSHA-Regulated Substances

All laboratories shall ensure that employees' exposures to hazardous substances do not exceed the permissible exposure limits (PELs) specified by OSHA in 29 CFR 1910, subpart Z. In addition, initial monitoring of employees' exposure to any substance regulated by a standard (29 CFR 1910.1001-1101) which requires monitoring shall be conducted if there is reason to believe that exposure levels for that substance routinely exceed the action level (or in the absence of an action level, the PEL). If this initial monitoring reveals that an employees' exposure exceeds the action level or the PEL, the exposure monitoring provisions of the relevant standard shall be complied with.

If a PEL has not yet been established for a study material, alternative acceptable exposure standards (e.g., TLV®, REL, WEEL) shall be used (Refer to Section III.A.1.). In situations where there is no known exposure standard for a proposed test article, a suitable interim exposure standard based on current toxicology and industrial hygiene literature shall be established when feasible.

b) Formaldehyde Monitoring in Histology, Pathology, and Anatomy Laboratories

Histology, pathology, and anatomy laboratories must comply with the formaldehyde OSHA standard, 29 CFR 1910.1048. The use of formaldehyde in all other laboratories shall be carried out in accordance with the OSHA formaldehyde standard.

Histology, necropsy, tissue storage, and tissue trimming operations shall be conducted in a manner that employs engineering controls to ensure that airborne concentrations of formaldehyde do not exceed 0.75 ppm as an 8-hour TWA or 2 ppm as a 15-minute STEL. Monitoring shall be performed to evaluate exposure levels (both TWA and STEL) of workers potentially exposed to formaldehyde hazards. If the results of initial monitoring indicate exposure levels exceeding either 0.5 ppm as an 8-hour TWA (action level) or 2 ppm as a 15-minute STEL, additional monitoring must be performed. If the TWA exceeds the action level, sampling must be repeated every 6 months. If the STEL exceeds 2 ppm, sampling must be repeated annually. The repeat sampling may be discontinued when 2 consecutive sampling rounds are below the STEL and action level as described in the OSHA formaldehyde regulation, 29 CFR 1910.1048. In addition, all other provisions of the OSHA formaldehyde standard must be adhered to.

c) Test article/Positive Control Monitoring

Exposure monitoring shall be routinely conducted where both test article and controls are handled and when the test article/positive control has an established exposure standard such as the PEL, TLV®, REL, or WEEL of 10 ppm or less, or 0.1 mg/m3 or less. This exposure monitoring shall be performed at least once during initial dose preparation, once during initial dose administration, and at the midpoint of the study or every six months. Where there is no known exposure standard for a proposed test article, the contractor shall perform exposure monitoring at the same frequency stated above. Determination of exposure and adoption of controls shall be based on a pre-determined interim exposure standard, when feasible.

5. Occupational Medical Surveillance

An occupational medical surveillance program is to be implemented to cover personnel who will be working with study test article or animals. The frequency of the surveillance as well as the scope of the medical examination shall be specified in the laboratory's Health and Safety Plan. Persons who are required to wear respirators must obtain written medical clearance from an occupational health service provider for use of this equipment.

6. Injury and Incident Reports

A record shall be kept of all injuries or illnesses, including animal bites. In addition any record of an OSHA recordable incident shall include a full description of the incident, the test article/positive control involved, the medical attention required, any remedial actions taken, and planned follow-up to minimize the likelihood, or eliminate the potential for, reoccurrence (if pertinent). Copies of such incident reports shall be forwarded to NTP.

The Project Officer shall be notified IMMEDIATELY if a serious (as defined by OSHA) accident or incident occurs.

All occupational injuries and illnesses shall be recorded and reported according to the OSHA recording system.

B. TEST ARTICLE/POSITIVE CONTROL HANDLING AND SAFETY POLICIES

1. Receipt/Handling/Storage

A log shall be maintained which will include the date of test article receipt and a continuous balance of the remaining amount of test article.

Weighing of the test article and/or positive control shall be done using the smallest quantity needed. An analytical balance shall be used whenever possible to preclude the need for handling large amounts of chemical. This balance shall be placed at all times in an effective laboratory hood or a vented enclosure exhausted to the outside (see Section III.C.3.). Protocols shall be designed to use the minimum possible quantities of "neat" chemical in preparing solutions.

A non-breakable, secured secondary container shall be used for transfer of any test article and/or positive control.

Volatile test articles shall be handled properly (e.g., keeping lids on container when not in use, segregating from unintended contact with heat or high pressure, etc.) and stored in an area with adequate ventilation that is directly vented to the outside. All other test articles shall be stored in a secured, designated storage area(s). However, flammable liquids must be stored in a non-vented flammable liquid storage cabinet (see Section III.E.1.).

2. Hazard Communication

a) Training

Personnel who handle (receive, store, weigh, dilute, transport, package, or administer) hazardous agents shall be provided with written material and trained on the associated hazards of these agents including the contents of the Material Safety Data Sheet (MSDS). This training shall be conducted by the HSO or a program approved by the HSO and shall be properly documented. Training shall include the recommendations for handling carcinogens. In addition, training in accordance with the requirements of applicable regulations shall be conducted.

b) Labeling

Warning signs and labels shall be used wherever test articles are used or stored (e.g., on primary and secondary containers, affixed to entrances to work areas, refrigerators, and on containers holding hazardous waste). These signs and labels shall be conspicuous (especially for containers to minimize handling) and shall indicate the presence of suspected carcinogenic, mutagenic, and other hazards, as required by OSHA.

c) Health and Safety Documents
The contractor must have available for each study agent and positive control health and safety documentation that includes, but is not limited to, the supplier’s MSDS*, which includes information on the material's hazards, properties and appropriate control measures. All employees handling the study material and/or positive control must be trained regarding the contents of the agent-specific health and safety data document.

MSDS shall be accessible at all times at designated locations known by the employees.

- If a chemical is produced for a user outside of the laboratory then the laboratory is required to develop an MSDS.

C. ENGINEERING CONTROLS

1. General Facility Requirements

Safety showers, drench hoses, and eyewash stations shall be located throughout the facility as required by local, state, and federal regulations and must be located in close proximity to where potentially hazardous chemicals are stored or used. Only plumbed eyewashes are permitted.

2. Isolation and Access Restriction

a) General Requirements for Access Restriction

An isolated, posted, restricted access laboratory (or laboratories) separate from other laboratory facilities shall be designated for unpacking, storing, weighing, and diluting of test articles and/or positive controls; and where necropsy, tissue trimming, tissue processing, embedding, microtoming and staining are performed.

Administration of test article and positive controls shall be performed in a limited access area with its air supply under negative pressure with respect to connecting laboratories and hallways. This shall be a separate laboratory from the area described above for areas dedicated to unpacking, storing, weighing, and diluting.

Each lab shall have a room inspection program providing monthly checks of the air flow directionality. Relative pressures of laboratory areas shall be checked monthly with smoke tubes to verify that air flows from relatively clean to relatively dirty areas. Monthly inspections shall be documented.

A record shall be kept of all personnel entering/exiting any limited access area(s).

b) Requirements for Barrier Systems

The dose preparation area shall be isolated from general traffic. This may be accomplished by locating the dose preparation area within the animal facility limited access barrier system, or by establishing a separate limited access area for dose preparation. If the latter approach is used, all areas into which laboratory workers may bring used protective equipment (including gloves, shoes, head covers, and clothing), respirators, and/or containers of dosed feed or water shall be behind the barrier. Also, any hallways used by workers for reaching the shower facility shall be considered to be behind the barrier (e.g., limited access area).

Personnel who enter the dose preparation area, or an area requiring a complete set of clean protective clothing and equipment (e.g., a disposable laboratory suit, safety goggles, disposable gloves with permeation-resistant properties specific to the test article, disposable boots, disposable shoe covers or sneakers or rubber boots, and disposable head covering), must shower out prior to leaving the barrier facility at the end of the day.

Within the shower facility, the "clean" and "dirty" sides must be physically separated by the shower or by another physical barrier. The facility design and procedures shall be arranged so that it is not necessary to require entering the clean side prior to showering and to prevent returning to the dirty side after showering (e.g., to store or retrieve items such as shoes, towels, respirators, etc.).

Each laboratory shall have a room inspection program providing monthly checks and documentation of the air flow directionality. Relative pressures of laboratory areas shall

be checked monthly with smoke tubes to verify that air flows from relatively clean to relatively dirty areas.

c) Facility Design for Barrier Systems

Air exhausted from dose preparation areas involving the particulate form of the test materials shall be passed through HEPA filters. If volatile chemicals are handled, charcoal filters shall also be used. These filtration systems shall be periodically monitored and maintained and personnel performing maintenance shall wear the protective clothing described for neat test article handling. (See Section III.D.1.a.)

The relative location of external air intakes and exhausts for both local and general ventilation systems must be arranged to minimize the risk of re-entrainment of exhaust air. Documentation (e.g., schematic diagram) shall be provided to NTP indicating the location of intakes and exhausts, stack height, discharge velocities, as well as the direction of prevailing winds. No weather caps or other obstructions shall be in the path of vertical discharge.

Within the barrier facility, walls, floors, and ceilings shall be sealed around all incoming and outgoing pipes, conduits, and other utilities to prevent release of contaminated material to surrounding areas. Animal rooms and dose preparation rooms shall be constructed of wall, floor, and ceiling materials which form chemical- tight surfaces. Animal room doors shall include windows to permit observation of workers within each room.

Emergency power generator systems shall be in place and emergency generator maintenance and testing shall be documented.

3. Hoods and Vented Enclosures

Where not superseded by requirements in this section, all work shall conform to the current edition of the Laboratory Ventilation Standard, Z 9.5, published jointly by the American National Standards Institute and the American Industrial Hygiene Association. Effluent exhaust concentrations shall not exceed federal, state, and local air pollution emission requirements.

a) Hood/Enclosure Operations and Requirements

The following operations, unless otherwise noted below, shall be performed in a laboratory hood or other enclosure:

- All dose preparation operations (e.g., weighing, premix, micro encapsulation, mixing of dosing solutions), as well as diluting, or administering (gavage, dermal, intra-peritoneal injection, inhalation chamber administration) of study materials/positive controls
- Test article weighing in laboratories (e.g., analytical laboratories)
- Transfer/filling of dosed feed containers
- Unpacking, analysis, and other handling operations involving test article/positive control or other hazardous agents
- Necropsy, tissue trimming, tissue processing, and staining
- Handling tissues, fluids, and exhaled air collected from animals for evaluation
- Cage and feed container dumping
- Plastic-backed absorbent matting shall be secured inside of any hood wherever the test articles and/or positive controls (including dilutions) are being handled. After

each working session in the hood, or sooner if there is known contamination, this matting shall be disposed of as hazardous waste.

NOTE: Operations that cannot be performed within a laboratory hood or other enclosure due to the size of the containers or equipment will be conducted using other engineering controls (e.g, local exhaust, enclosed systems), administrative controls (e.g., restricted access during operations), additional PPE, or a combination of controls that will provide equivalent protection of employees. The determination of appropriate controls will be made by the Health and Safety Officer.

(1) Hoods for Weighing, Diluting, or Administering Test Articles/Positive Controls

Laboratory hoods for diluting and administering test articles and/or positive controls (including gavage, dermal, intra-peritoneal injection and dosed feed hoods) shall provide sufficient contaminant and containment capture velocities (an average air flow velocity of 100 $\pm$ 20 fpm at the operating sash height with no individual point less than 80 linear feet per minute or greater than 120 linear feet per minute unless it can be demonstrated by testing, e.g., yearly use of smoke candles, that values greater than 120 fpm provide adequate capture and do not cause turbulence). In addition, face velocities of balance enclosures shall be at least 50 fpm.

Biological safety cabinets used for dilution or administration of toxic agents shall re-circulate no more than 30% of their air.

(2) Exhausted Enclosures/Hoods for Automatic Tissue Processing/Staining.

An effective exhausted enclosure or hood for automatic tissue processing or staining machines with exposed solvent systems shall provide sufficient capture velocities, e.g., 50 fpm minimum, as evaluated by a combination of velometer and smoke tube tests. Exhausted enclosures for automatic processors having exposed solvent systems shall be provided with a fire protection system and/or emergency power backup.

(3) Exhausted Enclosures/Hoods for Necropsy, Tissue Trimming, Manual Tissue Processing, and Manual Staining.

An effective exhausted enclosure or hood for necropsy, tissue trimming, manual tissue processing, and manual staining as well as for all handling operations involving tissues, fluids and exhaled air collected from animals considered to be contaminated with test article and/or positive control shall provide capture velocities of 80 $\pm$ 10 fpm (with no individual point less than 70 fpm or greater than 90 fpm unless it can be demonstrated by testing (e.g., yearly use of smoke candles) that values greater than 90 fpm provide adequate capture and do not cause turbulence).

b) Hood/Enclosure Venting

Hoods and glove boxes used for weighing, diluting, or administering test articles/positive controls shall be exhausted to the outside.

Effluent exhaust vapor from sample oxidizers and/or analytical instruments (e.g., gas chromatograph, atomic absorption spectrophotometer) shall be vented to the outside.

Motors for hoods and enclosures exhausted to the outside shall be mounted outside the building such that all ductwork shall be under negative pressure.

Re-circulation of air from local exhaust systems into occupied spaces shall not be permitted. The only exception to this will be for dosed feed container-filling hoods, cage dumping hoods, or vented enclosures for studies involving non-volatile, solid test articles. If re-circulation is desired in this case, the air discharged from hoods or vented enclosures must be equipped with HEPA filtration to clean air prior to its discharge to the study room. The HEPA filter shall be disposed of as hazardous waste. (See Section III.G.2.)

c) Hood/Enclosure Monitoring

Exhaust enclosures shall be smoke tested using smoke tubes to demonstrate no leakage of smoke out of the enclosure during normal operating procedures.

All ventilation systems shall be routinely monitored. Laboratory hoods and all other local ventilation enclosures shall be quantitatively monitored on a quarterly basis. For studies of 90 days or less duration, each hood or vented enclosure shall be verified within 45 days prior to the beginning of the study unless monitoring data indicate a different frequency.

The sash height at which the face velocity has been measured shall be marked on each hood along with the date of the last measurement, the measured flow, and name of the person performing the monitoring.

The Health and Safety Officer shall maintain records of ventilation system checks. The records shall indicate for each hood, room, and area, at a minimum, when air was tested, what was found, who conducted the test, and what equipment was used.

D. PERSONAL PROTECTIVE EQUIPMENT SELECTION

1. Selection

a) Operations Involving Handling of the Neat Test Article/Positive Control and in Animal Rooms.

Where the neat test article/positive control is stored, weighed, in dose formulation rooms and in animal study rooms, or areas into which personnel directly exit when leaving animal study rooms (e.g., dirty side of the barrier) the following minimum personal protective clothing shall be worn at all times:

- Disposable full-body Tyvek® (or equivalent) suit and disposable head covering, unless Tyvek® suit includes a hood
- Gloves

  If chemical-specific gloves cannot be identified, two pairs of dissimilar, disposable gloves (e.g., N-Dex® or equivalent, PVC, latex, natural rubber) shall be worn when handling test article/positive control (as neat material or in formulated doses). Both pairs of the two dissimilar gloves shall be changed after any known chemical contact and/or after every two hours of handling test article/positive controls or dose formulations.

- Respirator

  Appropriate NIOSH-approved respirators shall be worn.

- Eye Protection

  Splash-proof safety glasses, goggles, or other eye protection specified by OSHA and ANSI.

- Footwear

  Disposable shoe covers, disposable boots, or facility-dedicated rubber boots.

b) Operations Not Involving Neat Chemical/Positive Control
For laboratory operations not involving the handling of neat test article/positive control (e.g., chemical analysis, histology, tissue trimming, and necropsy on the clean side of the barrier), the following shall be worn:

- Single pair of disposable gloves

- Laboratory coat

- Splash-proof safety glasses, goggles, or other eye protection specified by OSHA and ANSI

c) Animal Barrier "Clean" Corridor

All staff entering the clean corridor with the intention of entering animal rooms must follow PPE requirements as defined in section D.1.a.

All staff entering the clean corridor for purposes other than entering animal rooms must wear disposable suits, scrubs, lab coats, or other launderable clothing dedicated to the facility; disposable head and shoe covers.

2. Respiratory Protection

Where specific engineering controls (e.g., vented enclosure for test article/positive control weighing) have been demonstrated to be effective in controlling exposure levels, the need for respiratory protection shall be determined by the Health and Safety Officer.

The Health and Safety Officer in accordance with OSHA regulations and NIOSH Respirator Decision recommendations shall select suitable, NIOSH-approved, task-specific respirators. Where Air Purifying Respirators (APR) are used (e.g., with gas/vapor and particulate combination cartridges), written provisions shall describe when cartridges are to be changed and the logic used to make this determination. The date and time of installation shall be marked on all cartridges. Where air supplied devices are used, breathing air is to be analyzed periodically to ensure that the quality of air meets human breathable air standards. Personnel who are required to wear respirators shall be medically cleared, trained, and mask-fitted before they are allowed to wear the respirator.

A respirator program that meets the requirements of OSHA 29 CFR 1910.134 shall be implemented for routine and emergency use of respirators.

Any respirator cartridge used during a clean-up of spilled chemical shall be disposed of as hazardous waste.

3. Usage and Storage Practices

All protective equipment used in a particular laboratory shall be stored in accessible and convenient locations as dictated by the barrier design or procedures.

Disposable protective clothing shall not be worn out of the laboratory/test work area where neat chemical is handled.

Work clothing shall be removed upon exit from the laboratory on a daily basis.

Previously used disposable clothing shall not be reused.

Non-disposable items are to be stored in covered containers until washed. If washing is done by laboratory personnel, they shall wear gloves and disposable suits while handling contaminated items. If washing is done by an outside service, they shall be notified in writing that they are handling items with potential contamination.

E. FIRE SAFETY

NOTE: The facility and operations shall comply with applicable federal, state and local fire and building codes.

1. Storage and Handling

Flammable liquids shall be stored and handled in a manner that will reduce the risk of fire and/or explosion. This includes the following:

All non-working quantities of flammable liquids shall be stored in storage cabinets approved by Underwriters Laboratories or Factory Mutual, or in a designated flammable liquids storage room with suitable fire protection, ventilation, spill containment trays, and with equipment meeting the requirements of OSHA. In either storage arrangement, the flammable liquids shall be segregated from other hazardous materials such as acids, bases, oxidizers, etc.

Flammable storage cabinets shall not be vented unless required by a chemical- specific OSHA regulation or by local authorities. Metal bung caps shall be used in place of flash arrestor screens. If it is necessary that venting be provided, the following shall be adhered to: (1) Remove both metal bungs and replace with flash arrestor screens. The top opening shall serve as the fresh air inlet. (2) Connect the bottom opening to an exhaust fan by a substantial metal tubing having an inside diameter no smaller than the vent. The tubing shall be rigid steel. (3) Ensure that the fan has a non-sparking fan blade and non-sparking shroud. It shall exhaust directly to the outside where possible. (4) The total run of exhaust duct shall not exceed 25 feet.

Class I flammable liquids shall not be stored in conventional refrigerators/freezers. If flammable liquids must be kept at low temperatures, they shall be stored in Underwriters Laboratory (UL) listed/Factory Mutual (FM) Global approved refrigerators/freezers designed for flammable storage. In a potentially flammable or explosive atmospheric environment, only those explosion-proof refrigerators/freezers listed for Class I. Division 1, Group C and D, and listed by UL as a "Special Purpose Refrigerator and/or Freezer" shall be used. All explosion-proof refrigerators shall be labeled as such.

Whenever flammable liquids are stored or handled, ignition sources shall be eliminated. This includes the prohibition of smoking.

Flammable liquid transfer shall be done in the designated storage room or over a tray within an effective laboratory hood. In the former location, all transfer drums shall be grounded and bonded and shall be equipped with pressure relief devices and dead man valves.

Safety cans shall be used when handling small (e.g., no more than 2 gallons) quantities of flammable liquids, unless chemical purity requirements require otherwise (e.g., distilled-in-glass grade, etc.).

2. Fire Safety Equipment

   a) Fire Extinguishers

      Fire extinguishers shall be conspicuously located where they will be readily accessible and immediately available in the event of fire as required by local, state, and federal regulations. Placement of portable fire extinguishers shall conform to OSHA 1910.157. The specific type and size of extinguisher shall be selected with consideration for the hazards to be protected and the strength of the personnel who might use the extinguishers. For the majority of laboratory applications, water and aqueous film forming foam (AFFF) extinguishers shall have a capacity of 21/2 gallons. Dry chemical, carbon dioxide, and foam extinguishes shall have 20-30 pound capacity.

   b) Safety Showers

      Safety showers shall be located in the immediate vicinity of every laboratory where flammable liquids are stored/used. Fire blankets may be used if available.

3. Training

   All personnel shall receive training in fire safety. Course material shall include hazard awareness, proper techniques for the handling and storage of flammable liquids, and a briefing on the alarm system and emergency evacuation preplanning. In addition, "hands-on" training for appropriate personnel on fire extinguishers is encouraged.

F. EMERGENCY PROCEDURE

The written set of general safety policies shall include actions to be taken in case of fire and/or explosion. They will address personnel assignments, evacuation routes, and notification procedures. The National Fire Protection Association Life Safety Code, Number 101, and existing manual pull-box locations shall be considered when establishing means of egress.

A written set of emergency/evacuation procedures to be followed by all project personnel in the event of a spill or leak involving the test article and/or positive control shall be developed and posted in each laboratory. Personnel shall be instructed to call for appropriate help (e.g., in-house emergency group or poison control center) in case of an emergency. This plan shall address the storage, use and maintenance of emergency protective equipment.

The location and phone number of the nearest poison control center and any other emergency phone numbers shall be prominently posted in each laboratory.

Emergency protective equipment shall not be stored in the laboratory where test articles are stored and handled.

G. WASTE DISPOSAL/TEST ARTICLE SHIPMENT

1. Disposition/Shipment of Surplus/Residual Test Article
   The following practices shall be adhered to concerning the disposition of surplus/residual test article:

Thirty days prior to shipment, the contractor shall notify the NTP of their intention to ship surplus or residual test article, including the amount to be shipped, and complete details of the shipping procedures, including the contractor to be used.

Upon completion of testing and after receiving approval from the Project Officer, the contractor shall immediately ship excess quantities of test articles after the final bulk chemical analysis has been completed. In addition, a 100 gram aliquot of each batch of the test article is to be reserved and shipped separately to the designated NTP chemistry support contractor after the final bulk chemical analysis has been completed. For reactive chemicals, gases, etc. the Project Officer shall be contacted to determine if any test article is to be reserved and shipped.

The following requirements for packaging these test articles are made in order to minimize the possibility of exposure to personnel involved in the packaging, transportation, and receipt of these test articles. The requirements shall be consistent with the Department of Transportation (DOT) regulations (or IATA regulation for contractors outside the USA) as outlined in 49 CFR, parts 100 to 199.

Test articles shall be shipped in primary containers compatible with the physical and chemical properties of the substances that prevent contamination of the study material. Each primary container must be securely sealed to prevent leakage during transport. After being sealed, the exteriors of each primary container must be decontaminated and labeled with all pertinent information (including chemical name, lot number, amount, date, and source). Test articles that are gases or liquefied gases in cylinders shall be shipped without additional packing and according to appropriate transportation procedures.

All primary containers shall be sealed in double plastic bags to prevent leakage and exposure if broken, surrounded by absorbent material and placed in secondary containers. Larger amounts of liquids may be shipped in five-gallon metal drums which are individually packaged and which meet all DOT regulations. These five-gallon drums must be over-packed in larger drums with absorbent material, securely sealed, and fully labeled. All over packed drums shall be fully filled, securely sealed, and completely labeled on the outside.

Outside containers must be free from extraneous and ambiguous labels. Labeling must include a directional label to indicate the top, appropriate warning labels, e.g. SUSPECT CANCER AGENT, FLAMMABLE, and all required DOT labels and identification. All shipments shall be made in compliance with DOT regulations (or IATA regulations where applicable) and accompanied by a completed Shipper Certification Form for Hazardous Materials. A detailed packaging list must be placed on the outside of the shipping container identifying each chemical fully by name, amounts shipped, and lot numbers of each chemical. NTP shall be consulted if the quantity or type of substance to be shipped renders these requirements inappropriate.

2. Potentially Contaminated Material

All potentially contaminated material (e.g., dose formulations, bedding, used personal protective clothing and equipment, absorbent materials for handling test materials, disposable cages, lab ware, filters, respirator cartridges, etc.) shall be incinerated or disposed of in a licensed hazardous waste landfill, in a manner consistent with federal, state and local regulations. Animal carcasses, blood samples, animal tissues, or any other materials that are grossly contaminated with blood, including sharps and syringes, shall be collected and disposed of by incineration. The laboratory shall indicate whether it plans to fulfill this requirement with its own incinerator, or by use of a licensed waste disposal firm. If the laboratory's incinerator is to be used, specifications (e.g., temperatures and residence times), operating procedures, and information on licensing by local regulatory authorities shall be

provided to NTP for evaluation. If a contract disposer is to be used, complete information on the firm's licensing and hazardous waste transporter shall be provided.

Where data is collected in animal rooms using computer terminals, the terminal shall be decontaminated using a chemical-specific solution when removed from an animal room after each use. Terminals must be disconnected from any electrical power sources before decontamination, and care will be taken to ensure that any solvents used do not damage the plastic parts of the computer terminal.

Vacuum lines, including water aspirators, used when working with test article/positive control shall be protected with an absorbent or liquid trap and a HEPA filter.

IV. CHEMISTRY

A. GENERAL REQUIREMENTS

1. NTP will typically supply the test article.

2. NTP will typically provide procedures for bulk chemical analysis, dose formulation and dose formulation analysis. Modest modifications may be made to suit existing instrumentation.

3. Biological sample analysis may be included in selected toxicology studies. In some cases, the contractor's responsibility may be limited only to the collection and shipment of samples/tissue(s) for analysis by an NTP chemistry contractor. However, for some studies the Testing Laboratory shall be required to develop methods for determining the test article or metabolite in biological samples, analyze samples and provide an interpretation of the results.

4. Standard Operating Procedures (SOPs) shall be prepared for all chemistry, dose formulation, and biological sample analysis operations.

5. The results of all analyses shall be reported to at least three significant figures unless the analytical methodology limits the data to fewer significant figures.

B. BULK TEST ARTICLE CHEMISTRY

1. Bulk Test Article Receipt and Storage

   The test article(s) and frozen reference(s), as well as storage conditions for the bulk test article, will be supplied by the NTP. Whenever feasible, a sufficient quantity of the test article will be procured so that only one lot of chemical will be needed to complete all of the contracted study phases. Testing Laboratories shall plan to have adequate storage at the specified condition(s) for bulk test article(s). A use log of the bulk test article shall be kept and submitted as part of the raw data at the end of the study.

   If appropriate storage conditions for the bulk test article are not provided by NTP, it may be possible to use the manufacturer's data to establish the stability and thus the storage conditions for the bulk test article without the need for additional studies by the Testing Laboratory. If stability studies are required they are to consist of triplicate analyses of the bulk test article after storage at - 20, 5, and 25º C for two weeks in sealed vials that are protected from light.

2. Initial Identity and Purity

   Methods to confirm the identity and purity of the bulk test article are to be provided by NTP, and will generally involve two identity analyses and one purity determination.

   It shall be necessary to conduct re-analyses to confirm the purity of the test article while it is being used in the toxicology studies. The most efficient method for determining stability is to maintain a stable standard for comparison with the stored bulk test article. Therefore, upon receipt of each batch of test article, the Testing Laboratory shall remove enough samples for all reanalysis tasks (typically 15 X 1 gram samples). Each sample shall be placed in a glass vial with a Teflon-lined top that is tightly closed. All reference samples shall be placed in a freezer and maintained at –20º C ± 5º C for storage prior to analysis. Analyses of the bulk test article and the reference standard shall be run in tandem, so that the bulk chemical purity can be compared to that of the reference standard from the same batch. Each vial of reference test article shall only be used for one reanalysis. Any proposed repackaging of the test article by the contractor must be approved in advance by NTP. In specific cases, to be

identified by NTP, additional frozen reference samples may be required to be taken from each individual bottle or drum of test article supplied, for use in bulk reanalysis of those specific containers while they are in use.

3. Bulk Test Article Chemical Reanalysis

   The Testing Laboratory shall check the purity of the bulk test article at 24 ± 2 week intervals while the article is at the Testing Laboratory's facilities. The bulk test article re-analyses will normally require the use of only one purity analysis method. These bulk chemical re-analyses shall include an analysis within thirty days prior to the start of any study. Additional test article re-analyses shall include an analysis within thirty days after euthanasia of the last animal for studies greater than 30 days duration.

   The Principal Investigator shall immediately inform the NTP Project Officer via telephone or e-mail of any significant change in purity (e.g., a difference not explained by variability within the analytical procedure used) or appearance of the test article during the study and note it in the next Progress Report.

C. DOSE FORMULATION

1. Methods for formulating the test article shall typically be provided by NTP.

2. The stability of the dose formulations shall typically be established by the NTP. The dose formulations shall not be used beyond their stability period.

   If a stability study is to be the responsibility of the Contractor, the stability of the test article formulated with the dosing vehicle shall be conducted at the lowest concentrations specified for the toxicology study. If suspensions are to be formulated, the re-suspendability of the highest concentration formulation shall also be determined. Stability studies shall be run for a 42-day period, at 1 week intervals, with the dose formulations stored sealed and protected from light at -20, 5, and 25º C. Triplicate samples shall be analyzed. Stability determinations shall also be made under conditions that simulate the environmental conditions of dosing. Results of stability studies shall be reported to the COTR as soon as they are available by telephone and electronically.

3. An inventory of each dose formulation shall be maintained. A record of the formulation date and use date for each formulation shall be kept. This record, which shall be signed by the dose formulation supervisor, shall contain information on the quantities of dose formulation prepared and identifying numbers for both the test article and dosing vehicle.

4. Each time an aliquot of the bulk chemical is weighed and formulated with the vehicle, that formulation is defined as a BATCH.

5. One archival sample of each batch shall be set aside at the time of preparation for possible dose analysis checks and stored in individually labeled, sealed containers. All aliquots shall be stored at –20º C ± 5º C except for aqueous formulations that shall be stored at 5º C ± 3º C. The quantities for the archival samples are: approximately 50 mL for gavage and drinking water studies; 100 g for feed studies; and 25 mL for dermal studies. When a dose analysis of a formulation is planned, the analytical chemistry technician shall retrieve the archival samples from the storage area. Archival samples that are not selected for analysis shall be discarded as hazardous waste in accordance with federal, state and local regulations ninety days or more after preparation. (NOTE: Each time an aliquot of the bulk chemical is weighed and formulated with the vehicle, that formulation is defined as a batch. Therefore, for each dose prepared on each formulation day it is possible that two or more batches will be required.).

6. To ensure that homogeneous dosed feed or suspensions are prepared, homogeneity shall be checked prior to initiation of each study phase (e.g. range-finder or full study) at the highest and lowest doses, unless blend parameters (size and concentration) have not changed from the previous study. For feed studies, samples shall be taken for analysis from three different points in the blender (e.g. top left, top right, and bottom of the twin-shell blender; two samples per site). For suspensions, two samples shall be taken from the top, middle, and bottom of the container under simulated dosing conditions. All samples taken for homogeneity study shall be analyzed in duplicate. The formulation shall be considered homogeneous if the coefficient of variation of all samples taken for analysis is within 5%.

7. To prevent improper dosing of study animals:

    - The containers shall be labeled with the test article name, number, other identifying data and intended species. The inclusive dates of use must appear on the containers.

    - The labels on the containers shall be color coded for different dose groups, species, and sex (if different concentration used for species/sex).

    - Control and dosed formulations shall be stored separately from the bulk test article.

8. For dosed water studies, water for control groups is to be taken from the exact same source and at the same time as the water used for the treated group formulations. The control water and dose formulations are to be stored in carboys in the refrigerator until it is time to dispense the formulations to water bottles and transport them to the animal rooms. For dosed feed studies, feed for the control groups is to be taken from the exact same source and at the same time as the feed used for treated group formulations.

D. FORMULATION ANALYSIS

1. Formulation analysis methodology shall typically be provided by NTP. However, in some cases this may be the responsibility of the Testing Laboratory.

2. The Testing Laboratory shall validate the method over the proposed range of doses. If doses required for subsequent study phases are outside the validated range from those used in the initial study, additional pre-start validation studies and reports shall be required.

3. As a quality control check, formulations shall be analyzed periodically (see IV.E.) by the Testing Laboratory. The results of analysis for formulations shall be reported using the units specified in the study protocol. Analyses are to be carried out without the dose formulation personnel being told which formulations are scheduled for analysis.

4. Routine analyses of dose formulations shall be conducted using the following procedures:

    - Prepare two standard stock solutions of the chemical from independently weighed samples. Use these solutions to prepare at least six vehicle standards at concentrations bracketing the concentration range expected for the samples such that alternate standards are prepared from each stock solution.

    - Analyze the spiked vehicle standards and a vehicle blank. Duplicate analyses of spiked vehicle standards are not required.

    - Calculate the regression equation and the correlation coefficient for linear regressions or coefficient of determination for weighted linear regressions. Compare the regression equation with all previous equations. Any significant differences in slope or intercept

shall be explained. The correlation coefficient (r) must be > 0.99 and coefficient of determination (r^2) must be >0.98.

- Analyze the submitted dose formulation samples in triplicate.
- Use the regression equation sample responses and any dilution factors to compute the concentration of the unknowns.
- For chromatographic analyses, only single injections are required.
- Relative Standard Deviation will be calculated by determining the mean and standard deviation of each triplicate formulation analysis concentration. The standard deviation is divided by the mean and multiplied by 100% to obtain the relative standard deviation, which is also known as coefficient of variation (CV).
- A Q-test may be used to statistically identify outliers that may be rejected from the triplicate analysis sample data set. This is done by calculating the absolute value of the differences between the questionable result and its nearest neighbor and then dividing by the range (highest value – lowest value) of the entire set resulting in the quantity Qexp. The Qexp value is compared with the rejection critical value (Qcrit) that was obtained for the respective number of observations, and if Qexp > Qcrit, the questionable result may be rejected with the chosen degree of confidence (e.g., 90%).

5. When formulation analyses are required, analyses shall be completed prior to administering the dose formulations.

6. Precision and accuracy of the methods shall be such that values that deviate from the target concentration by more than 10.0% will be considered out of tolerance. There may be cases where a 10.0% tolerance limit cannot be attained and these will be addressed on an individual basis and must be approved by NTP. The cause of any deviation from the approved tolerance limit shall be discussed in the Monthly Progress Report. If the dose formulation is out of tolerance, the dose formulation shall not be given to the animals without NTP approval. Re-mixes shall be shown to be within the accepted tolerance limit before they are used for dosing. If a re-mix is necessary, aliquots will be analyzed according to the original procedure.

7. Reprocessing (diluting or adding test article to) out of tolerance dose formulations is not acceptable.

E. FORMULATION ANALYSIS REQUIREMENTS

1. <u>Studies for less than or equal to 30 days (e.g., teratology study, range-finding study)</u>

   a. All batches for the initial dose formulations of each dose group shall be analyzed to demonstrate the accuracy of the formulation procedure.

   b. Samples of the formulations shall be taken from the animal room according to the following scheme:

      (1) The samples shall only be taken from formulations for which dose formulation samples have already been analyzed.

      (2) The sample shall be taken on the last dosing day prior to the expiration date of the batch.

(3) The sample shall be taken at the end of the dosing day.

(4) The sample submitted for analysis shall be the residual formulation in the original dosing vessel. For drinking water studies the sipper tube assemblies are to be removed and the bottles capped. For dosed feed studies the contents of the feeders are to be emptied into clear, interferent-free containers. In addition, for dosed feed and drinking water studies, samples of the unused formulation from which feeders or bottles are filled shall be collected and analyzed along with animal room samples in determining animal room sample stability.

(5) Samples shall be taken from one sex of each species and each dose group (If dose groups for each sex are different then samples must be taken from each sex).

(6) Each sample shall be analyzed in triplicate.

The results of these analyses shall be compared to the results of the original analyses.

c. If the NTP has directed that the formulations are to be analyzed by another laboratory, the samples shall be labeled according to the example below, and a sample submittal form, also provided below, shall be prepared and included with the shipment. The laboratory is to be notified (by e-mail or phone) at least 24 hours prior to arrival of the shipment. Information regarding the carrier and tracking number is to be provided when available. In addition, appropriate return address information is to be included on the package. If the shipment will arrive during non-working hours or requires special handling or storage conditions, the laboratory is to be contacted at least 48 hours prior to arrival so that arrangements can be made to receive and handle the shipment properly.

2. Studies greater than 30 days and equal to or less than 90 days (e.g.,range-finding study)

a. All batches prepared for the initial, midway (if appropriate), and final dose formulations for each dose group shall be analyzed to again demonstrate the accuracy of the preparation procedures and analytical methods.

b. A homogeneity study is required for feed and suspension studies (See Section IV.C.5.).

c. In addition to the formulation room samples taken and analyzed at the beginning, midway, and end of the study, samples of these same preparations shall be taken from the animal room (upon completion of dosing) and analyzed as described in section IV.E.1.b. above.

3. Studies greater than 90 days (e.g., RACB, Modified One-Generation study)

a. All batches prepared for the initial set of dose formulations shall be analyzed. Thereafter, these analyses shall be carried out every 10 ± 2 weeks.

b. A homogeneity study is required for feed and suspension studies (See Section IV.C.5.).

c. At the beginning of the study, samples of all initial dose formulations shall be taken from the animal room for analysis as described in section IV.E.1.b. above. Thereafter, similar animal room samples shall be taken during every third scheduled analysis period.

4. Recommended Sample Label Format

Chemical Name:_____

Vehicle: _____

Concentration: _____

Identification: (optional)* _____

Dates Prepared:_____ Expiration:_____

Storage Condition:_____

* If a specific sample identification (e.g., Testing laboratory's unique identifying code) is required in a report, it can be placed here.

DOSE FORMULATION ANALYSIS SAMPLE SUBMITTAL FORM

DATE _____

NAME OF ORGANIZATION _____

RETURN ADDRESS _____

NAME OF SUBMITTER _____

TEST ARTICLE _____

CAS # _____

TYPE OF STUDY _____

SPECIES/STRAIN _____

VEHICLE _____

TEST ARTICLE LOT NO _____

DATE MIXED _____

Sample Identification	**Concentration**	**Approximate Amount Shipped***

* Minimum required:

Feed:	**100 g each (Blank - 200 g)**
Gavage:	**50 mL each (Blank - 150 mL)**
Water:	**50 mL each (Blank - 100 mL)**
Dermal:	**25 mL each (Blank - 50 mL)**

F. ANALYSIS OF DOSING VEHICLES

1. The Testing Laboratory shall perform analyses of dosing vehicles to confirm the identity and purity. The protocols for the analyses are given in Appendix 2.

2. Frequency of the analyses shall be as follows:

   a. Any batch of corn oil shall be analyzed for peroxides before it is first used and at bimonthly intervals thereafter while it is in use. All corn oil must be stored at 5º C or lower.

   b. Any batch of ethanol, acetone, methylcellulose or other vehicle shall be analyzed on receipt for identity and purity and then for purity every six months thereafter while it is in use. Sufficient material shall be purchased so that enough of a single lot is available for the entire study for which it is purchased. Ethanol (other than absolute) used for dose vehicle preparation must be demonstrated to contain less than 0.01 ppm benzene.

G. PRESTART CHEMISTRY REPORT

1. Prior to starting toxicity studies, a special report is to be submitted to the NTP to include the following items (where applicable for individual test articles):

   a. Information on the source of the bulk chemical as well as manufacturer stability, storage requirements, identity and purity data and a color picture of the bulk material compared to a color spectrum

   b. Confirmatory data on identity, purity, (including representative chromatograms and spectra of the test article and reference material, and if necessary, stability of the bulk chemical using methods developed by NTP or the Testing Laboratory as well as copies of the SOPs for the bulk chemical reanalysis methods

   c. Data on preparation, handling, stability (if performed by the testing laboratory), homogeneity, and analysis of dose formulations as well as SOPs

   d. Dose formulation analysis method performance evaluation procedures and validation data, including representative chromatograms or spectra (see Appendix 1)

   e. Health and safety procedures

2. Animals **shall not** be dosed until this report has been received and approved by NTP.

H. BIOLOGICAL SAMPLE ANALYSIS

1. For some studies, biological samples such as blood, liver or other tissues, may be collected for analysis of test article concentration. For some test articles, toxicokinetic studies may also be conducted. These are usually designed to: 1) determine the actual internal dose concentration; 2) determine the extent of bioaccumulation; 3) evaluate the effects of sex, age and long term exposure on specific toxicokinetic parameters. The data may be used to select dose levels, route of administration and vehicle for the toxicology studies and/or to evaluate the possible correlation of toxic effects with toxicokinetics/internal dose. Ultimately, the data should prove useful for risk assessment.

2. Biological sample analyses or toxicokinetic studies generally require the characterization of the blood/plasma concentration of the test article at specific times after dosing. The quantitation of test article in other tissues at specified time-points may be included as well.

3. NTP will specify whether the Testing Laboratory is to collect and ship samples to another NTP-designated laboratory for analysis, or is to collect and analyze the samples themselves.

4. For studies of reproductive and developmental toxicology, biological samples shall generally be shipped to another laboratory for analysis. However, if the Testing Laboratory is to analyze the biological samples and the anticipated concentration range of the analyte in the biological samples has previously been established, then the Testing Laboratory shall develop the quantitative analytical method and a method performance evaluation shall be conducted. The stability of the analyte in the biological matrix at a concentration approved by the NTP COTR shall then be established for the period of time over which the samples can be stored prior to analysis.

5. If the anticipated concentrations in the biological samples obtained in the planned studies are unknown, then the Testing Laboratory shall establish the expected concentration range by conducting a preliminary study. NTP will provide the design for this study (typically in the SOW). The Testing Laboratory shall then develop, but at this time not optimize, a bioanalytical method for quantitation of the parent chemical or its metabolites in the biological matrix.

6. Dose formulations for preliminary biological sample or toxicokinetic work are not to be analyzed. The dose formulation method development work is to consist of only feasibility studies that will entail the non-quantitative evaluations of homogeneity and syringeability. Interim data from those analyses are to be submitted to NTP for review and together with the contractor, a decision will be made as to:

   a. the need for additional preliminary work

   b. the required ELOQ for the biological analysis method

   c. the range over which the method is to be validated

   d. the length of time over which the stability study in biological matrices will be conducted

   The data from the preliminary study will be reported to NTP in a written document that includes:

   a. design of the preliminary biological sample analysis study and results

   b. bioanalytical method description

   c. detailed procedures for biological sample collection and processing

   d. description of dose formulation methods used

   If the decision is made to go forward with further biological sample analysis, and the test article concentration in blood or in other biological tissues is within a range for which quantitation is technically feasible, the Testing Laboratory shall validate the bioanalytical method over the appropriate concentration range. (See Appendix 1.) Details for requirements for the validation studies and subsequent analyses shall be supplied by the COTR and can also be found in the NTP Specifications for the Conduct of Studies to Evaluate the Toxic and Carcinogenic Potential of Chemical, Biological and Physical Agents in laboratory Animals for the NTP, Section IV.J.

I. DISPOSITION OF SURPLUS/RESIDUAL TEST ARTICLE

1. Thirty days prior to the shipment of the test article, the contractor shall notify the NTP of their intention to ship surplus or residual chemical, including the amount to be shipped. Shipment is to be made within 30 days after the terminal euthanasia for the last study for that test agent. (See Section III.G. for complete details.)

2. A completed Surplus Test Article Aliquot Transmittal Form (provided below) shall accompany shipments of aliquots and surplus test article. In addition to the surplus chemical, a 100 g aliquot of each batch of chemical is to be reserved and shipped. The chemistry support contractor is to be notified (by e-mail or phone) at least 24 hours prior to arrival of the shipment. Information regarding the carrier and tracking number is to be provided when available. In addition, appropriate return address information is to be included on the package. If the shipment will arrive during non-working hours or requires special handling or storage conditions, the laboratory is to be contacted at least 48 hours prior to arrival so that arrangements can be made to receive and handle the shipment properly.

SURPLUS TEST ARTICLE TRANSMITTAL FORM

DATE _____

NAME OF ORGANIZATION _____

RETURN ADDRESS _____

NAME OF SUBMITTER _____

TEST ARTICLE* _____

CAS# _____

Provide information below for each lot used.

Lot #	Date lot Received	Temperature lot stored	Amount of Test Article Returned	List study types for which lot used

* Use full NTP test article name and abbreviation.

SUMMARY OF CHEMISTRY REQUIREMENTS

Category	Type	Anticipated Frequency	Reporting Requirement	Comments
Bulk Analyses	Identity and Purity Analyses	Once on receipt	Prestart Chemistry Report	Methods provided by NTP
	Stability Study		Prestart Chemistry Report	
	One Purity Analysis	24 ± 2-weeks	Study Report	
Corn Oil Analyses	Peroxide Level Determination	Bimonthly	Study Report	Method is provided by NTP
Vehicle Analyses	Identity and Purity Analyses	Once on receipt	Prestart Chemistry Report	See Appendix 2 for NTP supplied methods
	One Purity Analysis	Every 24 ± 2 weeks	Study Report	
Dose Analyses Method Performance Evaluation	Dose Analysis	Once	Prestart Chemistry Report	Using methods provided by NTP validate before initiation of first animal study. If concs change in subsequent studies, additional MPE's will be required
Dose Analyses for Studies 30 days or less (such as a Teratology Study)	Formulation Room Dose Analyses	Initial only	Study Report	All batches at each dose
	Animal Room Dose Analyses	Initial only	Study Report	Each dose for each sex and species
	Homogeneity Study	Once	Prestart Chemistry Report	Prior to preparation of formulation designated for dosing (feed and suspension only); total of two samples from each of three sites
Dose Analyses for Studies greater than 30 days and equal to or less than 90 days (such as a multi-generation DRF)	Formulation Room Dose Analyses	Initial, Middle, and Final	Study Report	Same as for studies less than 30 days
	Animal Room Dose Analyses	Initial, Middle, and Final	Study Report	Same as for studies less than 30 days
	Homogeneity Study	Once	Prestart Chemistry Report	Same as for studies less than 30 days
Dose Analyses for Studies greater than 90 days (such as an RACB Study)	Formulation Room Dose Analyses	Initial and every tenth ± 2 wks	Study Report	Same as for studies less than 30 days
	Animal Room Dose Analyses	Initial and every 3rd scheduled formulation room analysis	Study Report	Same as for studies less than 30 days
	Homogeneity Study	Once	Prestart Chemistry Report	Same as for studies less than 30 days

V. LABORATORY ANIMAL MANAGEMENT AND TOXICOLOGY

A. ANIMAL FACILITY OPERATIONAL REQUIREMENTS

1. Humane Care of Rodents in NTP Studies

   a. Animals shall be anesthetized to alleviate pain in procedures that may cause more than momentary or slight pain.

   b. Animals with a) large masses or other conditions interfering with their eating and drinking, b) major injuries or ulcers related to husbandry and treatment, c) debilitating conditions or other conditions indicating pain or suffering as judged by the veterinarian or an experienced scientist shall be euthanized immediately to avoid further pain and distress. (See Appendix 3.)

   c. Moribund animals and animals scheduled for interim and final necropsies shall be euthanized by personnel trained in methods and techniques established by the AVMA 2007 Guidelines on Euthanasia (Formerly Report of the AVMA Panel on Euthanasia) or most current version http://www.avma.org/resources/euthanasia.pdf

      Approved methods include:

      - Anesthetic overdose (injection or inhalant)
      - Injection of euthanasia solution
      - Prolonged exposure to CO_2 gas in cylinders (for rodents greater than 10 days old)
      - Decapitation with surgical scissors with or without anesthesia for rodents up to 10 days of age

      Fetuses gestational day 15 to birth and neonates from birth to 10 days of age shall be euthanized by approved methods based on the needs of the study.

2. Facility Compliance

   a. The testing facility shall comply with the Animal Welfare Act of 1966 (P.L. 89-544) as amended in 1970, 1976 and 1985 and other applicable Federal, state and local laws, regulations and policies.

   b. The testing facility shall adhere to the principles outlined in the "Guide for the Care and Use of Laboratory Animals" (NRC, 2011) and the NTP Specifications.

   c. The testing facility shall have a PHS Assurance from the NIH Office of Laboratory Animal Welfare (OLAW), and shall be accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

   d. The testing facility must have a functioning Animal Care and Use Committee.

   e. The testing facility must have qualified Laboratory Animal Veterinarian(s) with appropriate education and training to ensure the care and health of the animals.

3. Emergency Notification Procedure

   a. Each contractor shall keep an Emergency Notification Procedure that shows who to notify in the event of various types of potential emergency situations. This procedure shall be posted in a prominent location and on pertinent equipment (e.g. refrigerators, freezers,

etc). Weekend duty personnel, in particular, shall be aware of its location. All personnel must read and initial, acknowledging they have read and understand the procedures.

b. In all cases, the Principal Investigator (or designated alternate, if the Principal Investigator is absent) shall be notified of emergency situations. The Laboratory Animal Veterinarian must be made aware of any emergency situation that impacts animal welfare.

4. Pest Control

Programs to control or eliminate insects, escaped or wild rodents, and other similar pests shall be in use before starting animal studies. Pesticides and traps may be used as necessary in conjunction with a strict program of sanitary maintenance. However, to prevent toxic effects in research animals and possible interference with experimental procedures, pesticides, including insecticide impregnated plastic materials, shall not be used in the animal rooms, feed and bedding storage areas, and any other areas of the facility where animals, cages, racks, feed, bedding, and water may be exposed to the pesticides (when used) either in particulate or in vapor form.

5. Sanitization of Equipment and Animal Facility Rooms

The sanitizing chemicals shall not contain essential oils, perfumes, fragrances, or any other chemicals expected to influence the metabolism of mammalian systems.

6. Movement of Animals Between Rooms

a. Studies (study animals) shall not be moved to a different room during the course of the study except for the reasons stated below:

- Specific statement in the protocol of the study requiring or permitting the move; such as for weaning, mating, crossover mating, or neurobehavioral testing.

- The physical condition (floor, walls, ceiling, fixtures, etc) of the room and/or its adjacent supporting area deteriorated to the extent that a safety hazard is judged to exist.

- The physical plant or ventilation equipment and ventilation, or lighting equipment and fixtures deteriorated to cause highly variable environmental conditions in the room;

- Change in physical factors in and around the study room, procedures to control an existing pest problem and/or procedures to control disease and microbial spread.

b. If a move is necessary, an attempt shall be made to accomplish this move as soon as possible to allow for sufficient acclimation prior to an important event such as clinical pathology evaluation, planned necropsy, etc.

c. When a study is moved to a new or different animal room due to physical conditions listed above in 6.a., the equipment to control environmental conditions, health and safety conditions, and the conditions to control disease and microbial spread shall be substantially superior in the new room when compared to the previous animal room.

d. Except in cases of emergency, approval for the move based on physical conditions shall be obtained from the NTP Contracting Officer's Technical Representative (COTR) in advance. There shall be a detailed procedure for this type of move and the study report shall include reasons and approvals for the move, date of the move, and detailed procedure of the move.

7. SOPs for Laboratory Animal Management and Toxicology

The test facility shall have specific SOPs for all laboratory animal management and toxicology procedures that address, but are not limited to, the activities listed below.

a. Technician training

b. Procedures for handling emergencies/disastrous situations in the animal facility

c. Testing and maintenance of emergency backup systems

d. Pest control procedures

e. Procedures for disease control and for prevention of microbial spread in the study facility

f. Sentinel Animal Program

g. Environmental conditions of study rooms including lighting

h. Sanitization of animal rooms before the receipt of animals

i. Sanitization of test facility and study rooms during the study

j. Sanitization of racks, cages, feeders, and watering system

k. Operation and maintenance of cage and rack washers

l. Watering System

m. Receipt and storage of feed including evaluation of nutrient and contaminant reports to satisfy the NTP standards

n. Receipt and storage of bedding including evaluation of physical quality from a randomly selected bag and contaminant report to satisfy standards

o. Feeding and change of feeders

p. Rack, cage, and bedding change

q. Rack and cage rotation

r. Receipt and examination of animals

s. Quarantine, health evaluation, and release of animals for study

t. Randomization of animals

u. Identification of animals

v. Weighing of animals

w. Observation of animals: Daily AM and PM check, detailed clinical observations

x. Evaluation of feed and/or water consumption

y. Handling of dead and moribund animals and criteria for moribund euthanasia

z. Custody transfer of animals from animal care/toxicology to necropsy/pathology for the interim and final euthanasia of study animals.

aa. Criteria for disposition of escaped animals

bb. Gavage dosing procedure

cc. Monitoring of sanitation practices

dd. Movement of staff, animals, test articles, supplies, and waste throughout the animal facility

ee. Procedures for mating rodents

ff. Care and handling of pregnant and lactating animals

gg. Recording signs of parturition and determining date of birth

hh. Procedures for examination/staining of the uterus for implantations and resorptions

ii. Handling, weighing, gender determination and conduct of clinical observations for rodent pups; evaluation of developmental landmarks (AGD, preputial separation, nipple retention, vaginal opening, etc)

jj. Procedures for culling of rodent pups

kk. Procedures for weaning of rodent pups

ll. Randomization of rodent pups for continuation on study

8. Emergency/Disaster Response and Management Plan
The facility shall have an emergency/disaster response plan specifically addressing the animal facility.

B. ANIMAL ROOM ENVIRONMENT

1. The ventilation system shall provide a minimum of ten complete changes of room air per hour without drafts. There shall be no recirculation of room air unless it has been treated to remove all particulates and toxic vapors by effective filters and where necessary, scrubbers to avoid spread of disease and to eliminate the recirculation of contaminants.

   An automatic recording and alert system shall be used to monitor the ambient conditions in each animal room. If a completely automated system is used, the probes to determine room temperature and humidity shall be in the exhaust for each room. If a free-standing, portable temperature and humidity recording system is used, the equipment shall be located near the room exhaust at a level of three to four feet from the floor.

   Each month the contractor shall record qualitative evidence of the correct direction of airflow in each animal room. Quantitative measurements of flow rate shall be made at least twice per year, once in the cooling and once in the heating season.

2. Temperature of the animal room for rodents shall be maintained at 72º F ± 3º F. The temperature shall not be below 69º F or above 75º F during the course of the study and shall be maintained with minimal fluctuations near the middle of the range. There shall be an

alarm system for warning of temperature fluctuations beyond the 69 to 75º F range. If the temperature is below or above the 69 to 75º F range, it shall be returned to the acceptable limits within two hours. The relative humidity of the animal room air for rodents shall be 35 to 65%. It shall not be below 35% or above 65% during the course of the study. If the relative humidity is below or above the 35 to 65% limits, it shall be returned to the acceptable limits within two hours. Thermometers and probes shall be accurate within 2º F or better. Accuracy of thermometers and hygrometers must be checked as often as necessary, but not less than at quarterly intervals. Animal room temperature and humidity results shall be reported as either means ± relative standard deviation or as time-weighted averages.

3. The animal rooms shall be windowless (except for window in door) and uniformly lighted, preferably by diffuse lighting with fluorescent lamps. The light cycle in the animal rooms shall be twelve hours light and twelve hours dark, with the timing of the light/dark cycles varying no more than ± 15 minutes from day-to-day. Appropriate means must be taken to prevent light from entering the animal room during the dark cycle. The light cycle shall be controlled by automatic equipment with monitoring of proper functioning at two to three day intervals. The NTP requires a uniform light intensity of 30 ± 3 foot-candles at 3.3 feet (1.0 meter) from the floor for normal lighting of the animal rooms. During the observation periods, for convenience of the technicians, the light intensity may be increased up to 45-55 foot-candles at 3.3 feet from the floor. To accomplish this lighting, the animal room may be equipped with two-stage lighting, both stages to be automatically turned off by an automatic timer. The first stage will be the normal lighting for the room and it will be wired to turn on and off by an automatic timer. The second stage will be to facilitate observation of the animals. The second stage shall be wired to be turned off manually when not needed for observations. In the event the second stage lighting is not turned off, the automatic timer shall turn off not only the first stage but also the second stage lighting at the set time. The lights shall not be turned off during the light (day) phase or turned on during the dark (night) phase except in case of emergency. Emergency power must be connected to the light timers/controls and to some lights of the animal room.

4. Procedures shall be in place to limit and reduce the noise inherent in the day-to-day operations of an animal facility, including separating the procedures that make the most noise, such as cage washing, from the animal rooms for reproduction and developmental toxicity studies. Noisy animals, such as dogs, shall be separated from reproduction and developmental toxicity studies. Likewise, production of excessive vibrations in animal housing areas shall be avoided.

5. The temperature and humidity ranges for rabbit rooms are as follows: 64º F ± 3º F and 50 ± 10% humidity.

C. DIET AND WATER

1. Diet

   a. Irradiated, certified NIH-07 or NTP-2000 open formula diet shall be used for rodents, unless a different diet is specified by the COTR. All diets used must be irradiated and the testing laboratory shall receive the diet from an NTP approved vendor. NIH-07 shall be used during periods of pre-mating, mating, pregnancy and lactation. NTP-2000 shall be used during study cohorts/phases that do not include mating, pregnancy or lactation, such as for 90-day cohorts after weaning. Diet shall be changed from NIH-07 to NTP-2000 at the time of weaning. Diet shall be stored at 70º F or lower, 50% relative humidity or lower, and in a well ventilated area stored raised above the floor, and away from the wall. Diet shall be used for no more than 120 days post milling. Records shall be kept of the diet used for each study and include the type of diet, milling date, batch number, dates used, whether pellet or powder, and the source. Each batch of diet used shall be analyzed for contaminants, protein, fat, fiber, ash, moisture and heat-labile nutrients such

as Vitamin A and thiamine. Lists of contaminants with maximum acceptable levels are given in Appendix 3. A copy of the analysis records shall be included in the study records and sent to the NTP Archives. It is the responsibility of the Testing Laboratory to verify that the diet meets the NTP standards prior to use. The testing laboratories may be required to ship a sample of the diet to the NTP or an NTP designated analytical laboratory for nutrient and contaminant analysis. Irradiated Purina 5K96 may be required for some studies and it is stable for 180 days.

b. Rabbits shall be fed NIH09 open formula diet or other approved feed obtained from an NTP approved vendor. The diet shall be certified and irradiated.

c. Diet must be supplied to the animals in a feeder. Unless otherwise specified, pelleted feed (wafer) shall be used for all routes of administration except dosed feed studies. Powdered feed (meal) shall be used for dosed feed studies.

d. Clean feeders with fresh food must be supplied to rodents at least once weekly. Rabbits shall be fed daily. Sufficient fresh food shall be provided as often as necessary to ensure support of normal growth and maintenance. Food hoppers for powdered feed shall not be filled to the brim (filling to less than 80% capacity will help to decrease the spillage). Rodents must have food ad libitum, unless specified otherwise.

e. Hoppers shall be dumped in a vented enclosure in the dirty cage wash area. Dirty feeders are to be soaked when necessary, and then washed in at least one cycle of 180º F water.

f. In order to avoid cross-contamination during washing, feeders used for all dosed feed studies shall be uniquely marked or labeled in order to identify the test article being dosed, each dose group, and the control group feeders.

2. Water

a. Municipal drinking water shall be supplied *ad libitum*. Water shall not be hyperchlorinated or hyperacidified. The NTP may specify a suitable water treatment procedure for special cases.

b. Laboratories must demonstrate that water provided for animal use meets US/EPA National Primary Drinking Water Regulations. Appendix 3 contains a list of additional water components and contaminants to be determined and assessed. To satisfy this requirement, the laboratory must provide analyses of water from an animal room (or a port outside the animal room that is contiguous with the animal room water line) or a composite from several animal rooms used for NTP studies at least once every 12 months (+/- 30 days). For dosed water studies, water used for such analysis is to be taken from the specific source used to make the dose formulations. If the laboratory performs any water treatment, details of this treatment must be provided. For laboratories new to NTP, an additional report shall be provided to the NTP within thirty days of contract award. The testing laboratories may be required to ship a sample of water to NTP or an NTP designated analytical laboratory for contaminant analysis once a year. Water analyses shall be performed by a laboratory qualified to conduct such studies on a local, state or interstate level.

c. When an automated watering system is used, the valve end shall be located outside the cage, which will require that a stainless steel grommet be affixed around the access port to the watering valve. Care must be taken to ensure that the animals can reach the valves, and that the valves are placed such that cages cannot be flooded in the event of a malfunction.

d. Water bottles may be used, although an automated watering system is preferred. Water bottles shall be of sufficient capacity so that no more than two per week per cage are routinely needed. Each cage shall be supplied at least twice weekly with a fresh sanitized water bottle, bottle cap, and sipper tube; twice weekly for group housed animals and weekly for individually housed animals. Dirty (used) bottles shall be exchanged for clean bottles, **never refilled and reused**. Water bottle stoppers shall be made of an inert material and bottles shall be located in a position to prevent the stoppers from being chewed by the animals.

e. Dosed-water studies

For dosed water studies, water for control groups is to be taken from the exact same source and at the same time as the water used for the treated group formulations. The control water and dose formulations are to be stored at 5°C in glass carboys until it is time to dispense the formulations to water bottles and transport them to the animal rooms.

In order to avoid cross contamination during washing, water bottles used for all dosed water studies shall be marked indelibly, inscribed or tagged with a permanent color coding so as to identify the test article being dosed (symbol may be used), each dose group, and the control group water bottles.

f. Washing Water Bottles

Bottles, bottle caps, and sipper tubes must be soaked and washed promptly. Water bottles must be washed in water of at least 180º F. Water bottles are to be:

- Washed with regular cage washing detergent using a brush apparatus, suitable for bottles being used; or,
- Washed in an automatic washing system wherein the water outlet for the bottle washing process is well within each bottle being washed.
- If the bottles are to be washed in a standard tunnel washer, each bottle at each dose (study water bottles only) shall be filled with tap water and rinsed twice prior to washing. During washing, control bottles and those from each dose group shall be kept separate from each other and not washed with bottles from other dosed water studies.

D. CAGING, RACKS, BEDDING AND FILTERS

1. Caging

a. Cages for the NTP studies shall be program and test article specific. Cages shall be returned to the same test agent to avoid possible contamination. Each cage containing animals shall always be identified with a cage label that includes the study, cage number and animal number(s) within that cage. The cage identification card shall be attached to the cage and shall be transferred along with the animal(s) to a new cage throughout the in-life portion of the study.

b. Polycarbonate cages shall be used in a suspended cage rack system for rodents, unless otherwise specified by the NTP. The racks shall have provisions for placing filter fabric on the shelf above the cages.

c. Rodents shall be caged together according to the weight-space specifications recommended in "The Guide for the Care and Use of Laboratory Animals." During the

quarantine period, animals shall be initially apportioned to cages as if they were in the upper weight range for a study. Thus it will not be necessary to redistribute them later to larger cages in order to remain within the recommended weight-space specifications.

d. Female rats and mice shall be housed up to five per cage. Male rats shall be housed up to three to five per cage depending on their size (weight) and male mice shall be housed individually. During the mating period, one male rat and one female rat shall be housed together, unless otherwise directed by the COTR, depending on study type. Pregnant animals shall be individually housed; there shall be no more than one dam with litter per cage. For group-housed rats, pregnant rats and rats with litters, the cages shall measure approximately 22" L, 12.5" W and 8" H. For group housed female mice, the cages shall measure approximately 12.5" L, 9.25" W and 6" H. For individually housed male or female mice, the polycarbonate, solid bottom cages shall measure approximately 9.25" L, 6" W, and 6.125" H. If an automated watering system is used S-shaped stainless steel watering manifolds shall be used to facilitate sanitization at the time of rack sanitization. The rack watering manifolds shall be flushed or drained at least once a day to prevent matter and bacterial accumulation. Rabbits shall be housed in mesh-bottom caging. Caging shall provide at least 3 sq ft/animal and shall be 16 inches in height.

e. Group housed animals shall be changed to a sanitized cage twice weekly and individually housed animals shall be changed to a sanitized cage once weekly, or as often as necessary to keep the animals clean and dry. Remaining cage groups shall not be combined. If cage changing becomes more frequent than the above schedule on a continual basis, the NTP COTR is to be notified as this might indicate a treatment-related effect.

f. Cages containing sentinel animals for the disease screening program shall be scattered throughout the racks in the animal room.

g. Dirty cages shall not remain in the animal rooms. After changing, they must be washed promptly in a machine that provides one rinse cycle of at least 180^{o} F water. The wash cycle shall include a detergent.

2. Racks

a. Stainless steel, suspended type racks shall be used, unless otherwise specified.

b. Racks shall be capable of being moved to the wash area for periodic machine sanitizing, or if they are fixed racks, sanitization must be provided for each of these racks.

c. Racks must be kept clean while in use and, in particular, the wheel surfaces must be cleaned 360º when the floor is being cleaned.

d. It is preferred that racks are run through a rack washer which includes one cycle of 180º F water, or they shall be moved to a wash area, hosed and washed using a suitable detergent, and hosed down under high pressure.

e. Racks shall be sanitized at least every other week.

At the time of sanitization of racks, the automatic water manifolds:

- Shall also be sanitized by flushing with hot water at 180^{o} F or higher for at least one minute preferably after flushing with warm detergent solution to remove organic matter, OR

- Shall be sanitized by flushing/exposure to a sanitizing solution (chlorine) for 30-60 minutes followed by flushing with water for at least 2 minutes.

f. Water manifolds on each rack shall be flushed daily (at least 60 seconds) and each watering valve shall be checked for proper water flow.

3. Bedding

a. For rodent studies, irradiated, heat-treated hardwood bedding that meets the NIH standards for physical quality and for chemical and microbiological contaminants is available from commercial manufacturers. (See Appendix 3 for maximum acceptable level of contaminants). The testing laboratories shall receive the bedding and the analysis data from an NTP approved vendor for each batch of bedding used. It is the responsibility of the testing laboratories to make sure that the bedding meets the NIH and NTP standards. The testing laboratory is not expected to perform additional analyses on the bedding. The testing laboratories may be required to ship a sample of the bedding to the NTP or an NTP designated analytical laboratory for contaminant analysis. Bedding is not used for rabbit studies.

b. The bedding shall be stored off the floor, away from the wall and in a fashion to prevent contamination.

c. Fresh bedding shall be supplied in clean sanitized cages as specified above.

d. Nesting material (e.g., crinkle paper) may be used as directed by the COTR.

4. Filters

a. Non-woven, synthetic fiber filters shall be used on the cages.

b. A fresh filter sheet shall be supplied at least every other week.

E. ANIMALS

1. Strain/Stock and Source

a. The CD-1 mouse or Harlan Sprague Dawley rat (Hsd: Sprague Dawley SD) shall typically be used in these studies unless otherwise specified by NTP. The New Zealand White rabbit shall be used in certain non-rodent studies. All animals shall be obtained from an NTP approved vendor.

b. Rodents and rabbits are considered acceptable models for studies evaluating toxic effects of chemicals. Data from these species are routinely used by regulatory agencies to conduct risk assessments.

c. For developmental and reproductive toxicity studies, time-mated female HSD rats shall be 11-12 weeks of age and 200-220 grams at mating unless specified otherwise by the protocol; male rats shall be at least 13 weeks of age at the time of mating. Time-mated female rabbits shall be 2.5 -4 kg (4-6 months of age) upon receipt.

d. When animals are shipped by air, they shall be transported from the airport to the testing laboratory without delay. All shipments, regardless of route, from the NTP approved suppliers containing dead, moribund, or otherwise unsatisfactory animals must be reported immediately to the NTP COTR and Laboratory Animal Management. If a shipment of animals is not received, the NTP Laboratory Animal Management shall be notified as early as possible to assist in tracing the shipment.

2. Animal Receipt and Quarantine

a. Shipping cartons/crates and the filter fabric shall be examined for damage that occurred during transit. Animals that arrive in damaged cartons shall not be used. Thoroughly wipe the entire outside of the shipping cartons with an appropriate disinfectant. Disinfected shipping cartons are to be separated from shipping cartons that have not been disinfected. Do not spray on and around the shipping cartons. Disinfected, unopened shipping cartons shall be taken directly to the door of the specific animal room, but shall not be taken into the animal room. During unpacking and transfer of animals to cages, the animals and the person in the animal room removing the animals from the carton shall not come in contact with the outside surfaces of the shipping cartons. Time-mated animals used in perinatal studies may be shipped to the testing laboratory over a period of several days. Animals from the same shipment and supplier or shipments received within several days from the same supplier for the same study may be maintained together. The Laboratory Animal Veterinarian shall examine the animals within 24 hours of arrival to assess their health status. In the case of receipt of time-mated animals, examinations may need to be conducted sooner, depending on the protocol.

b. Animals shall be quarantined/acclimated up to a maximum of fourteen days under conditions simulating those in the study situation. Newly received animals may be used in a study after a 3-day acclimation period but shall remain under quarantine until released. At the end of the quarantine/acclimation period the animals shall be examined and released from quarantine (if healthy) by the Laboratory Animal Veterinarian.

1) Animals shall be housed one to five per cage by sex to simulate the housing to be used during the testing phase of the study.

2) If automated watering is used in the testing facility, it shall be used during the quarantine/acclimation period. Group housing is permitted for up to 7 days at the beginning of the quarantine period for acclimation to automated watering.

3) The animals shall receive the same textured feed (meal or pellets) from the same source during quarantine as they will receive during the study period.

4) Animals shall be observed for signs of normal eating, drinking and behavior as well as for any untoward signs of transit stress or other problems.

c. The health of the animals shall be assessed during the quarantine period.

1) Unsuitable animals, as determined by the Laboratory Animal Veterinarian, shall be discarded and not placed on study. Before discarding, at least five/sex of these unsuitable animals (all if less than five) in the worst condition shall have a health assessment. If there are no unsuitable animals, randomly select healthy animals (five per sex).

2) For studies using vendor-supplied time-mated animals, the testing laboratory shall receive 10 age matched non-mated females to be used for health assessment.

3) Animals undergoing health assessment shall be bled for serology assessment of rodent pathogens, euthanized, examined for internal and external parasites, and necropsied. The Laboratory Animal Veterinarian and/or Veterinary Pathologist shall examine all necropsied animals grossly for disease. Diagnosis of lesions seen at necropsy shall be confirmed by histopathological examination and/or microbiological culture.

THE PRESENCE OF INTERNAL OR EXTERNAL PARASITES AS WELL AS ANY UNUSUAL DISEASE CONDITIONS RECOGNIZED IN QUARANTINED ANIMALS SHALL BE REPORTED TO THE COTR AS SOON AS DETECTED/DIAGNOSED.

3. Animal Assignment to Study

   a. During the last 1-3 days of quarantine/acclimation, or as specified in the protocol for time-mated animals received from the supplier, animals shall be assigned to test/control groups following formal randomization routines.

   b. If sufficient healthy animals are available, these animals shall be randomly assigned to groups by body weight. The weight distribution range of rodents selected for the study shall be as narrow as possible, no more than ± 10% from the mean body weight (by sex) of all the animals available for the study at randomization. If it is necessary to use a few animals outside the ± 10% range, approval by the COTR shall be obtained. For rabbit studies, the weight distribution range shall also be as narrow as possible.

   c. It is necessary that in all studies the body weight means of the groups within a gender be close to each other. A randomization procedure shall be used to achieve this objective after outliers are removed from the group of animals available for study. Extra animals not assigned to test groups shall be removed from the study room and accounted for in the raw data. Extra animals may be used as sentinel animals if necessary or for training of technical personnel.

4. Animal Identification

   a. The method of identification shall be approved by the NTP. Animals shall be uniquely numbered within a study. Consecutive numbering shall be used within dose groups (or litters). Once a number is used for an animal in a study, it shall not be repeated in the same study. Tattoo at the base of the tail with black pigment is the preferred method for all studies with albino and pigmented rats and mice. Double strength black pigment is recommended for tattooing pigmented mice. Some tattoos may fade and they may have to be retouched during the course of a study. The scheme used for identification shall become a part of the raw data.

   b. The tail, or other body part bearing the identification marks, shall be fixed with the tissues at necropsy.

   c. There must be a method to track parentage of every animal born in a study. Limb or paw tattoo may be used to identify pups.

F. ANIMAL DISEASE SCREENING PROGRAM

1. Study Requirements

   An Animal Disease Screening Program is directed by the NTP. Testing Laboratory participation is mandatory. Studies greater than 6 weeks in duration require the use of additional animals to be used as sentinels. Sentinel animals must be clearly marked as sentinel and used only as sentinel animals. Sentinel rats and female mice shall be group-housed and the male sentinel mice housed individually. Sentinel cages shall be randomly placed throughout the racks of control and treated animals. Sentinel animals shall be received with study animal shipments from approved vendors. Sentinels may be the culled offspring of control study animals in generational studies, or may be the offspring of sentinel animals mated to produce additional sentinels. The COTR shall inform the testing laboratory of the source of the sentinel animals.

a) Sentinel Animals Examination

Sentinel animals are examined 4 weeks after the study start date and at the end of each study (serology and examination for internal and external parasites). If the study duration is over 6 months, serology shall be conducted at approximate 6 month intervals. Ten animals are used at each time point (five per sex; or 10 of the same sex if only one gender is available at a required timepoint such as a study that starts with time mated females).

1) Blood shall be collected (and serum separated) from sentinel animals at designated time points. Animals shall be examined for internal and external parasites, necropsied, and examined for gross lesions following the collection of blood samples. Blood is collected via cardiac puncture or abdominal vessels (at necropsy) under CO_2 /O_2 anesthesia. Lesions shall be processed for histopathologic examination and the results reported to the NTP COTR as soon as they are available. The presence of internal or external parasites shall be reported to the COTR immediately.

2) If serum samples are collected from animals used for clinical laboratory studies and the Animal Disease Screening Program, then the anesthetic, site of bleeding and blood collection technique specified for clinical laboratory studies shall be used. Serum samples are shipped to an NTP designated Rodent Disease Diagnostic Laboratory for disease screening.

3) For mouse studies, fecal samples shall be collected from five male and five female sentinel mice for evaluation for *Helicobacter spp* infection by an NTP-approved rodent disease diagnostic laboratory. The protocol shall indicate the collection time for these samples (which shall occur during the latter half of the study).

b) Weighing

It is not necessary to weigh the sentinel animals or measure their food or water consumption at any time during the study. If all animals for a study, including the sentinels, are pooled for randomization purposes, then initial body weights would be measured.

c) Moribundity/Mortality Checks

The sentinel animals shall be checked at the same time the regular animal observations are made to assure they are alive. No program notes are necessary and the animals need not be palpated or otherwise handled unless a moribund or dead animal is found. If a sentinel animal is found dead, the death shall be recorded. If a moribund sentinel animal is found, a blood sample shall be taken before sacrifice. The sample shall then be frozen and included with the samples from the next scheduled sampling. The sample, in essence, becomes part of the next scheduled sampling.

d) Pathology

Any sentinel animals lost to the study shall not be replaced. Sentinels that die or are euthanized during the course of a study shall receive complete necropsies and shall be examined to determine if disease or internal or external parasites are present. Selected histopathology (to include all lesions and grossly abnormal organs) shall be done on dead and moribund sentinel animals and the results reported to the NTP as soon as they are available, but no later than ten work days after the dead or moribund animal was found.

One copy of the Individual Animal Necropsy Records (IANRs) (electronic or paper form) for the dead and moribund sentinel animals shall be submitted to the Contract Coordinator of the NTP. If TDMS is used for the study, data from these forms shall not be entered into TDMS. The slides, blocks, and tissues shall be labeled with a non-TDMS label containing the experiment, the animal identified as sentinel, date, tissue, etc. The slides, blocks, and wet tissues of the sentinel animals shall be sent to the NTP Archives along with the rest of the tissues from the study.

2. Collection, Processing and Shipping of Disease Screening Specimens

   a. A volume of at least 0.1 ml (100 ul) of diluted serum at a 1:5 dilution (1 part serum and 4 parts normal or phosphate buffered saline) is required for serology assays. The sera shall not be heat inactivated or treated in any way. Sera shall be stored frozen until shipment.

   b. The testing laboratory shall submit serum samples for screening for the presence of antibodies to rodent pathogens. The screening tests shall include but not be limited to the following:

      Mice: Pneumonia virus of mice (PVM), reovirus type 3 (Reo 3), Theiler's encephalomyelitis virus (GD VII), ectromelia, Sendai, mouse hepatitis virus (MHV), mouse parvo virus (mouse minute virus and mouse parvo virus), lymphocytic choriomeningitis virus (LCM), EDIM, mouse norovirus and *Mycoplasma pulmonis*.

      Rats: PVM, Sendai, rat coronavirus-sialodacryoadenitis virus (RCV-SDA), rat parvo virus (RPV, RMV, KRV, H-1), Theiler's encephalomyelitis virus (GD VII), and *Mycoplasma pulmonis*.

   c. Rats shall be tested for *Pneumocystis carinii* once during the study at a time specified by the COTR.

   d. For mice only, 2-3 fresh fecal pellets per animal shall be collected with clean gloves or sterile forceps and placed in individually labeled sterile containers (one container per each individual animal/cage) for determination of *Helicobacter spp*. Gloves or forceps shall be changed between animals/cages. Fecal pellets can be collected directly from the animal or from the cage. Samples shall be shipped by an overnight delivery service but do not need to be frozen or chilled.

   e. All serum and feces shall be submitted to the NTP-approved Rodent Disease Diagnostic Laboratory in containers that are labeled legibly with a waterproof, indelible permanent marker. Each label shall correlate with a corresponding line on the Disease Screening Specimen form (provided by the NTP COTR). A separate sample form shall be included for each species. Vial lids shall be tightly sealed to prevent leakage. Individual wrapping or clustering in groups of three to five vials with rubber bands, envelopes or plastic bags can help to prevent excessive moving during shipment. Serum samples shall be shipped frozen with ice packs in an insulated container. Fecal samples shall be shipped at ambient temperature or lower. All disease screening specimens shall be shipped via an overnight delivery service Monday through Wednesday only.

   f. All specimens shall be submitted to the NTP designated Rodent Disease Diagnostic Laboratory. The COTR will provide the address of the diagnostic laboratory.

G. SPECIAL REQUIREMENTS FOR GAVAGE ADMINISTRATION

1. In gavage studies, for each dose group, the concentration of the dose formulation will remain constant throughout the study, with the required dose provided by varying the volume administered based on animal body weight. The total volume of material given per animal per treatment shall not exceed 5 ml/Kg for rats, or 10 ml/Kg for mice without consultation with and written approval of the COTR. The volume selected must remain constant throughout all studies for a test article.

2. All animals of a gavage study shall be dosed during a "consistent, specified time" of the morning on each treatment day. The "specified time" shall be approved by the COTR. An entire dose group is to be dosed before moving to the next group. The treatment sequence of control and dose groups for each treatment day shall be randomized to avoid a control first and high dose last bias. For studies where animals are dosed more than once per day, details shall be provided in the protocol.

3. Documentation that each animal was dosed on each treatment day is to be recorded and submitted with the study files.

H. REQUIREMENTS FOR STUDIES WITH A MATING COMPONENT

1. Rodents shall be paired 1:1 in the late afternoon (e.g. after 3 pm). The female shall be moved to the male's cage. Sibling matings are to be avoided.

2. Vaginal cytology slides shall be prepared in a fashion so they may be permanently retained.

3. Confirmation of mating is defined as an in situ plug or presence of sperm in a lavage sample. Cage plugs are not considered to be definitive evidence of mating, but can be used as supportive information (e.g. estimating the gestation day of an apparently pregnant rat).

4. Gestation day 0 (GD 0) is defined as the day evidence of mating is noted.

5. Calculated reproductive indices

   Mating Index: Number of confirmed mated females / number of cohabiting pairs
   Fertility Index: Number pregnant / number of cohabiting pairs
   Fecundity: Number females with at least one live pup / number pregnant
   Littering index: Number of females delivering / number of cohabited pairs

VI. CLINICAL PATHOLOGY

A. CLINICAL PATHOLOGY ASSESSMENTS

The laboratory shall be capable of performing the following clinical assessments/measurements in a satisfactory manner:

1. Hematology

   The laboratory shall be capable of performing the following hematology measurements using automated or semi-automated systems (impedance or laser-optic instruments) optimized and validated for rodent species.

   a. Erythrocyte count

   b. Hemoglobin concentration

   c. Hematocrit (Packed cell volume)

   d. Mean corpuscular volume

   e. Mean corpuscular hemoglobin

   f. Mean corpuscular hemoglobin concentration

   g. Leukocyte count

   h. Leukocyte differential count

   i. Reticulocyte count

   j. Platelet count

   k. A spun (manual method) hematocrit (Packed cell volume) must be performed.

   l. A morphological assessment (microscopic evaluation) of erythrocytes, leukocytes and platelets must be performed and documented. Nucleated erythrocyte (nRBC) counts (nRBC/100 leukocytes) must be reported.

   m. Instead of an automated leukocyte differential count, a leukocyte differential count, determined by microscopic examination of a Wright's-type stained blood smear and identification of at least 100 leukocytes (manual method), is acceptable. A manual differential count must be performed if the automated leukocyte count or leukocyte differential count generates instrument errors or abnormal cell counts/distributions/findings.

   n. Instead of an automated reticulocyte count, a reticulocyte count determined by microscopic examination (manual method) of a supravitally-stained blood smear (for example, new methylene blue) is acceptable. The manual reticulocyte count must be reported as an absolute number based on the proportion of reticulocytes in 1,000 erythrocytes or by use of a Miller disc.

      Platelet, reticulocyte and leukocytic cell counts will be expressed as absolute counts. The raw data will be determined by electronic or laser optic methods. When a manual leukocyte differential count is required (see Section VI.A.1.m.), absolute leukocyte cell counts may be derived by calculation using the instrument-derived total leukocyte count

and the microscopically-derived percentages obtained for the individual cell types. The reporting of data based upon percentages, estimates and manual (i.e. hemocytometer) counts is not acceptable.

At those times during NTP studies when blood samples are collected for hematologic analyses, the volume of packed red blood cells (VPRC or HCT) will be determined by manual (spun) micro methods. This procedure is in addition to the measurement (or calculation) of the hematocrit (HCT) performed by the hematology analyzer. Results of the manual determinations will be included with those of automated analyses in the data submissions. The plasma column in each microhematocrit tube will be inspected for the presence of hemolysis and a positive finding will be recorded and reported with these data.

2. Clinical Chemistry

The laboratory must be capable of performing the following serum clinical chemistry measurements using automated or semi-automated systems optimized and validated for rodent species.

a. Total protein concentration

b. Albumin concentration

c. Urea nitrogen concentration

d. Creatinine concentration

e. Alanine aminotransferase activity

f. Sorbitol dehydrogenase activity

g. Alkaline phosphatase activity

h. Total bile acid concentration

i. Glucose concentration

j. Creatine kinase activity

k. Cholesterol concentration

l. Triglyceride concentration

3. Urinalysis

The laboratory must be capable of performing the following urinalysis/urine chemistry measurements using manual, automated or semi-automated systems optimized and validated for rodent species.

a. Urine appearance

b. Urine volume

c. Urine specific gravity or osmolarity

d. Microscopic assessment of urine sediment

e. Urine protein concentration

f. Urine glucose concentration

g. Urine creatinine concentration

h. Activities of urine enzymes as specified for individual studies (e.g., N acetyl- β-glucosaminidase, lactate dehydrogenase, alkaline phosphatase, aspartate aminotransferase and gamma glutamyl transferase)

B. CLINICAL PATHOLOGY LABORATORY REQUIREMENTS

1. The clinical laboratory scientist is responsible for all phases of sample collection, handling (including transportation, recording, evaluation and preparation), analysis and storage. The training or approval of personnel to assume these tasks is also the responsibility of the clinical laboratory scientist. The clinical lab scientist must review and sign off on data at the end of each day, prior to discarding unused samples.

2. The laboratory shall have in place all equipment necessary to perform the above listed tests at a minimum.

3. The laboratory shall have routine capability to collect and analyze blood, serum and urine samples from rats and mice at a level of sixty samples for each study day. Non-routine situations may require collecting and analyzing sixty to one hundred twenty samples per day.

4. SOPs for performing the above referenced clinical tests shall be made available to the NTP for review and approval. The SOPs shall be accompanied by documented performance capability and ability to interpret the results. Each Testing Laboratory shall maintain clinical pathology historical control data for each sex and species used. Historical control data shall be made available to the NTP for review upon request.

5. Each laboratory performing clinical laboratory tests for the NTP shall have written quality control procedures (i.e., SOPs) that are followed and shall subscribe to a proficiency testing program. In-house quality control procedures include scheduled equipment maintenance and calibration and cumulative records of performance utilizing normal and abnormal control reference materials or samples. Cumulative records of proficiency program testing results shall be maintained. Prior to approval to conduct clinical laboratory tests, six-month cumulative data shall be submitted for NTP evaluation. The data shall be in graph or tabular form.

6. The laboratory must follow the Westgard rules for rejecting sample runs. (Westgard, J.O., Barry, P.L., Hunt, M.R., A multi-rule Shewhart chart for quality control in clinical chemistry. Clin. Chem., 27:493-501, 1981.) The laboratory shall have written procedures for the Westgard rules and describe the criteria utilized for the determination of sample run acceptability. For quality control data, the source of control materials, the mean and a measure of variability used in testing control materials to determine acceptability of sample runs must be maintained. If the mean and the measure of variability of commercially obtained controls were established in the Testing Laboratory, both in-house and manufacturer's values shall be maintained. The laboratory shall have written procedures for documenting incidents and deviations from protocol and SOPs. The degree to which such deviations and incidents may influence the clinical laboratory measurements shall be identified. Examples of such deviations and incidents include: technician error, variation in reagent lots, equipment drift or failure, and the rejection of data of sample runs based upon the concurrent analysis of control samples. Regardless of incident type, documentation of rejection and repeat of individual samples or sample runs must be made along with corrective action.

7. Whenever a test article specific protocol calls for hematology, clinical chemistry, etc., the blood samples shall be obtained in random order (not by dose group) for a given sex and species. The samples shall be run in that same random order in the clinical laboratory. The laboratory shall have written procedures for collecting and processing specimens in a randomized order. This requires an appropriate scheme for identifying and tracking specimens throughout all procedures.

8. Blood collection procedures shall be clearly identified. Blood collection sites, procedures, and anesthetics used shall be defined for interim as well as terminal sampling. Unless otherwise specified in the protocol, all blood samples from rats and mice shall be collected from the retro-orbital plexus/sinus using a carbon dioxide/oxygen mixture as the anesthetic. Sample volumes taken at interim bleeds shall not exceed 2.0% body weight for rats and 2.5% body weight for mice. Animals shall not be fasted prior to sample collection. For dermal and gavage studies, at each collection time point, animals shall be treated for a minimum of two consecutive days (within 24 hours) prior to sample collection. Animals are not to be treated on the morning of collection for these routes, unless the protocol requires it. Blood samples for analysis in the clinical pathology laboratory shall be collected from the appropriate animals the morning of sample collection during a 3-hour period. This includes samples for analysis of routine hematology, clinical chemistry, methemoglobin, urinalysis, and hormone variables. Methods for harvesting serum and plasma shall be described in written procedures. All males and females of a given species shall be treated the same number of days before collection of samples and all animals of a sex shall be bled on the same day. Overnight urine collection procedures must be clearly explained. As part of the demonstration of capability to collect blood and harvest serum, each laboratory must submit a listing of the total volumes per animal of whole blood, serum, and plasma that can be routinely obtained from rats and mice of three weeks, six weeks and six months of age, utilizing the retro-orbital bleeding technique. Interim, as well as terminal sacrifice volumes, shall be listed.

9. If unsuitable blood samples are obtained from individual animals, those animals shall not be re-bled on subsequent days to fill the data gap. If the data gaps are significant, the NTP COTR will determine if and when it may be necessary to re-bleed all animals.

10. Automated hematology measurements, blood smear preparations and methemoglobin determinations shall be made within 6, 2 and 0.5 hours of sample collection, respectively. Constituents in serum (or plasma, if specified) shall be assayed the same day of sample collection. Samples collected for routine hematology assays (EDTA) shall not be stored on ice before analysis. During the period when the samples are not being assayed, they shall be kept tightly sealed at 4º C. With specific permission from NTP, samples (e.g., serum) may be frozen at –20º C or colder for subsequent analysis. The freezing of samples for storage prior to the performance of routine assays is not acceptable.

11. The laboratory shall have sufficient facilities for frozen storage of biological samples at –60º C or below. Such samples may be retained for up to six months following NTP receipt of the relevant study report(s). Stained and cover-slipped peripheral blood, reticulocyte, and bone marrow smears shall be appropriately identified (same as for histology slide identification) and packaged for delivery to the NTP Archives at the conclusion of each phase of the study.

C. REPORTING REQUIREMENTS

1. Submission of Preliminary Clinical Pathology Results

Summary tables as well as individual animal data results for all sample collections of routine chemistry assays, automated hematology analyses, methemoglobin, hormone analyses, and urine chemistry determinations shall be submitted to the NTP COTR and Pathology Coordinator **within 7 calendar days** of sample collection. White blood cell differentials,

reticulocyte counts and morphologic evaluations of blood smears are to be included in the final report.

Data to be reported include original and repeat sample assays for individual animals. Copies of raw data may suffice for part of this requirement. It is not necessary for these data to be subjected to internal quality assurance prior to submission to the NTP. Summary and individual animal data are to be tabulated and organized by species, sex, and treatment group following the general format provided in Section XII.

2. Final Study Report

Summary data shall be included in the results section of the final study report. Individual animal data are to be included as an appendix to the final study report. Data shall be organized by species, sex and treatment group. Notations of any observation and/or action taken to confirm or explain atypical data points are to be included (example: diluted and reanalyzed to confirm or establish values that exceed linearity of assay, or sample reanalyzed to confirm low values). Relevant comments concerning sample quantity (QNS) and quality (example: lipemia, hemolysis, icterus, etc.) are to be included. Measures of central tendency in tabular form shall be reported for the measurements made at all time-points. The methodology used to obtain samples and measure analytes must be described in Materials and Methods Section of the study report. Interpretation of the biological significance of the results shall be presented in the Discussion Section of the study report and shall include correlations between clinical laboratory findings and anatomic pathologic changes and/or clinical signs exhibited by the study animals.

NTP CLINICAL PATHOLOGY STUDY INFORMATION SHEET

Laboratory Name:		
Study:		
Type: (example: RACB, Teratology, etc.)		
Time Point: (example: 5 day, 21 day, 90-day)		
Route of Administration: (example: gavage, dosed feed)		
Species:		
Sex: (circle) Male Female		
Date Collected:		
Date Analyzed:		
Anesthetic:		
Site of Sample Collection:		
	Equipment Used for Analysis	Technician(s) Performing Analysis
Hematology:		
Clinical Chemistry:		
Other: (example, hormone analysis)		
Comments:		

VII. PATHOLOGY

Data derived from developmental and reproductive studies focus on three main areas: (1) fertility and reproductive performance, (2) prenatal development, and (3) postnatal development. Various protocol designs are used to examine one or more of these areas in a study. The main body of this section provides information regarding necropsy and handling of tissues and slides from parental and post-natal animals in a study. Information on processing and examination of fetuses can be found in Section VIII.

A. CAPABILITY

Scheduled necropsies shall be performed in the presence of and under the supervision of the pathologist assigned to the study. The study pathologist shall examine the study protocol-required tissues from all necropsied animals in that study, including early death or interim sacrifice animals, and shall perform histopathologic interpretation on all study animals (this does not include fetuses unless required by the protocol). Additionally, the study pathologist may be invited to attend the Pathology Working Group review of the pathology data for that study at NTP.

B. NECROPSY

1. Necropsy Requirements and Sequence

A necropsy shall be performed on all protocol-required animals from any treatment group that dies or is euthanized during the study. If a breeding pair does not produce offspring, these animals shall receive a necropsy and histopathologic examination to determine the cause of the unsuccessful breeding. Sentinels that die or are euthanized during the course of a study shall receive complete necropsies with veterinarian present if possible. Escaped animals or animals inadvertently removed from animal room are not to be necropsied.

Scheduled necropsies and necropsies of moribund animals shall be initiated as soon as possible (within five minutes) after an animal is euthanized in order to minimize post-mortem autolysis. Unscheduled early death animals shall be necropsied as soon after death as possible. Ideally, dead animals shall be refrigerated for no longer than eight hours prior to necropsy and shall not be frozen. Unscheduled necropsies shall be performed in the presence of a pathologis. An effort shall be made at necropsy to establish the probable cause of death, e.g., gavage error, accidental death, infectious disease, treatment-related toxicity, developmental-anomaly, etc.

Necropsies on animals younger than PND 90 must be performed when the animal reaches the specified age at which it is to be euthanized without exception (e.g., PND 28 animals scheduled for euthanasia must be euthanized and necropsied on PND 28). Animals age PND 90 and older may be euthanized and necropsied within 2-3 days of the specified age, such as PND 90 $\pm$ 3 days. For logistical reasons, it may be necessary to stagger the start of an experiment so that necropsies can be performed at the required times.

Within a generation, necropsies shall be performed on one randomly chosen male or female control animal first, then a randomly chosen male or female high dose animal, followed by randomly chosen male or female mid- dose and low-dose animals if possible. This rotation shall be continued until all animals have been necropsied.

A necropsy for reproductive studies includes an external examination of the animal including all body orifices with special attention paid to the genitourinary system and mammary glands; examination of external genitalia and genital orifices as well as all internal organs (Table 1); and weighing and fixation of all organs/tissues listed in Table 2.

The following general guidelines are spelled out so there is no confusion:

For organ weights, organs shall be weighed to the nearest 1.0 mg, except liver and kidneys, which shall be weighed to the nearest 10.0 mg and epididymides, adrenals and ovaries to the nearest 0.1 mg. Organ-to-body weight ratios shall be calculated if required.

Sections of tissues collected at necropsy and saved for histopathology shall be fixed at a thickness not to exceed 0.5 cm unless otherwise noted.

Tails or other body parts that have been used in any way for animal identification during the in-life phase of the studies shall be saved in formalin with the animal tissues. If ear tags or other identifying methods are used, these too shall be saved in formalin with the animal tissues. For archiving purposes, all residual wet tissues shall be stored in NBF.

Publication quality color images of selected representative gross lesions in target tissues shall be prepared. All unusual lesions shall be photographed. All images are the property of the NTP. Each image shall be identified with contract number, chemical name, data management system reference number, sex, treatment group, animal number, generation, and description/diagnosis.

2. Necropsy Procedure
(Parental and Postnatal animals)

All gross lesions shall be described by the supervising pathologist and recorded using the terminology in the NTP Pathology Code Table (PCT) inclusive of morphologic lesion; anatomic site; size or volume in millimeters or milliliters; number; shape; color; consistency as appropriate. All gross lesions shall be recorded in the raw data entered directly into the computerized data management system or on the Individual Animal Necropsy Record (IANR) paper form as directed by the COTR. The IANR paper form and instructions for completing the IANR paper form are included in Appendix 4. Each gross abnormality shall be correlated with a gross and/or microscopic diagnosis.

During necropsy, extra attention shall be paid to the genitourinary system, both the external genitalia and genital orifices as well as internal organs. All abnormalities (e.g. malformations, lesions, etc.) must be precisely described and recorded, including the location and extent or size of the abnormalities noted (e.g., the location of the urethral opening for hypospadias) and whether the abnormality is unilateral or bilateral and complete or partial. Use of descriptive terms (ie, small or enlarged –with measurement) is preferred over interpretive terms (ie, atrophy) unless there is certainty regarding the lesion diagnosis. Table 1 shall be used to ensure that the genitourinary system has been carefully examined for abnormalities. Care must be taken when evaluating these lesions because some test compounds may result in agenesis of all or part of any of these organs and the absence of all or part of an organ must be properly recorded. Additionally, some lesions or malformations may be difficult to detect (e.g., hypospadias in a young animal), so these organs must be carefully examined. Generally histology does not need to be performed to confirm gross malformations.

At necropsy, the abdomen shall be examined for retained nipples in males and missing nipples in females and the number and location of each shall be recorded. In males, the prepuce shall be examined for incomplete preputial separation or clefting and the phallus for clefting, vaginal pouch, exposed os penis, hypospadias, or variation in size. The prepuce shall be retracted to examine the phallus for abnormalities. In females, the phallus shall be examined for clefting or variation in size, and the vagina for agenesis, the presence and patency of the opening, and vaginal thread. These and any other lesions or malformations that are identified shall be recorded.

Table 1. Approach to Characterize Developmental Endpoints, Lesions and Malformations in Offspring

MALES

Anogenital Distance (measure)

Nipples (enumerate, record location)
- Retained
- Inverted
- Other

Prepuce
- Cleft prepuce
- Incomplete preputial separation
- Other

Phallus
- Agenesis (complete or partial)
- Size (small, swelling/enlargement)
- Cleft phallus
- Vaginal pouch
- Exposed os penis
- Hypospadias
- Other

Testes (note if findings are unilateral or bilateral)
- Agenesis
- Size (small, swelling/enlargement)
- Fluid or blood filled
- Descended or undescended
- Location (i.e., abdominal or extravisceral)
- Presence/absence of cranial suspensory ligament
- Other

Epididymis (note if findings are unilateral or bilateral)
- Agenesis (cauda, corpus, caput, entire epididymis)
- Size (small, swelling/enlargement)
- Abnormal fluid or blood filled
- Other

Gubernaculum (note if unilateral or bilateral)
- Elongation (>20mm)
- Absence
- Other

Vas Deferens (note if findings are unilateral or bilateral)
- Agenesis (complete or partial)
- Distention (fluid or blood filled)
- Ectopic vas deferens (abnormal route or attachment)
- Other

Prostate (note if findings are unilateral or bilateral)
- Agenesis (ventral, lateral, or dorsal lobe; entire prostate)
- Size (small, swelling/enlargement)
- Abnormal fluid or blood filled
- Other

Seminal Vesicles and Coagulating Glands-Separate Entries (note if findings are unilateral or bilateral)
- Agenesis (complete or partial)
- Size (small, swelling/enlargement)
- Abnormal fluid or blood filled
- Other

Cowper's (Bulbourethral) Glands (note if findings are unilateral or bilateral)
- Agenesis (complete or partial)
- Size (small, swelling/enlargement)
- Abnormal fluid or blood filled
- Other

Levator Ani Bulbocavernosus Muscle Complex (LABC) (note if findings are unilateral or bilateral)
- Agenesis (complete or partial)
- Size (small, swelling/enlargement)
- Other

Urinary Tract
- Hydronephrosis (unilateral or bilateral)
- Hydroureter (unilateral or bilateral)
- Urolithiasis
- Hematuria or other discoloration of urine
- Other

Preputial Glands

FEMALES

Anogenital Distance (measure)

Nipples (enumerate, record location)
- Missing
- Other

Phallus
- Agenesis (complete or partial)
- Size (small, swelling/enlargement)
- Cleft phallus
- Other

Vagina
- Agenesis (complete or partial)
- Opening (patent, not present)
- Persistent vaginal thread
- Other

Uterus
- Agenesis (complete or partial, uterus unicornis)
- Size (small, swelling/enlargement)
- Hydrometrocolpus
- Other

Ovaries (note if unilateral or bilateral)
- Agenesis
- Size (small, swelling/enlargement)
- Follicular cysts
- Visible corpora albicans (enumerate)
- Other

Presence of Male Accessory Sex Organs/Tissues (confirm histologically)
- Testis, ovotestis or epididymis
- Prostate
- Seminal vesicles
- Coagulating glands
- Cowper's glands
- Levator ani bulbocavernosus muscle complex

The external examination of the carcass and measurement of the anogenital distance (AGD), if required, shall be completed prior to incising the skin. After the external examination, the left and right 4^{th} and 5^{th} mammary glands are to be collected if required by the protocol. In PND 90 and older animals, only the left and right 4^{th} mammary glands shall be collected. If required, a whole mount shall be prepared from the left mammary glands (see Appendix 6). The right mammary glands shall be collected in the same manner as the left, but shall be placed in a cassette and fixed in NBF for paraffin embedding. When collecting mammary glands, the lymph node MUST be included in all specimens. It is important for the pathologist to know the orientation of the mammary gland specimens when evaluating the whole mounts and histological specimens.

After the external examination and collection of the mammary glands (if required), incise the abdomen to expose the abdominal organs. Examine the organs *in situ*, recording and describing any abnormalities, paying particular attention to the organs of the genitourinary tract. Be sure to examine all animals for agenesis (partial or complete, unilateral or bilateral) of any organ and females for the presence of male organs, and males for the presence of female organs. Remove, examine, and weigh (if required) the internal organs listed in Table 2.

Table 2. Organs/Tissues Collected in Reproduction Studies and Their Fate

Organ/Tissues[a]	Weigh	Fate	Fixative
Left Testis	Yes	Freeze[b]	
Left Epididymis	Yes	Freeze[c]	
Left Cauda Epididymis[c]	Yes	Freeze[c]	
Right Testis[d]	Yes	Fix	Modified Davidson's
Right Epididymis[d]	Yes	Fix	Modified Davidson's
Dorsolateral Prostate	Yes	Fix	Modified Davidson's
Ventral Prostate	Yes	Fix	Modified Davidson's
Seminal Vesicles and Coagulating Glands (together)	Yes	Fix	Modified Davidson's
Preputial Glands [e] (weigh together)	Yes	Fix	NBF
Paired Cowper's (Bulbourethral) Glands[e]	Yes	Fix	Modified Davidson's
Levator Ani Bulbocavernosus (LABC) Muscle Complex[e]	Yes	Fix	NBF
Left Ovary	Yes	Fix	Modified Davidson's
Right Ovary	Yes	Fix	Modified Davidson's
Uterus/Cervix/Vagina[f]	No	Fix[f]	NBF
Retained Nipples[g] (if present)	No	Fix	NBF
Mammary Glands (for paraffin embedding)[h]	No	Fix	NBF
Mammary Gland Whole Mounts[h]	No	Fix	See Appendix 6
Adrenal Glands (weigh together)	Yes	Fix	NBF
Liver	Yes	Fix	NBF
Kidneys (weigh separately)	Yes	Fix	NBF
Thyroid Gland (fix prior to weighing)	Yes	Fix	NBF
Pituitary	No	Fix	NBF
Known Target Organs	Yes[i]	Fix	NBF
Gross Lesions	Yes[i]	Fix	NBF

[a] Additional tissues may be collected in some studies. Tissues expected to be affected during development may be excluded from examination in the F0 generation in some studies. Refer to the study protocol for specific instructions. See Appendix 6 for instructions on collecting and trimming non-reproductive organs. Unless otherwise stated, fix additional tissues in NBF and stain with H&E.

[b] Freeze at -70/-80º C for later assessment of testicular homogenization-resistant spermatid head counts for adult males.

[c] Prior to freezing, the left cauda epididymis and fluid must be kept at 37°C for accurate assessment of sperm motility. After removal of sperm, the left cauda epididymis shall be frozen with the caput and corpus for determination of caudal sperm concentration for adult males.

[d] Testes and epididymides collected for histopathology must be fixed in Modified Davidson's fixative for 24 hours then either processed and embedded immediately or transferred to 70% ethanol for a maximum of 72 hours after which time they must be processed and embedded.

[e] These tissues shall be grossly examined in all necropsied animals. However, they will be collected, weighed, fixed, and examined histologically only if triggered by study findings of malformations, abnormalities, or other lesions consistent with anti-androgenic activity.

[f] The uterus/cervix/vagina of each adult female shall be mounted on cardboard prior to fixation.

[g] If retained nipples are present in males, one retained nipple from each male with this finding shall be collected for histopathologic confirmation of nipple tissue.

[h] Mammary gland whole mounts and paraffin embedded mammary gland tissue sections from males and females may be required in some studies. Refer to Appendix 6 for instructions on tissue collection, as well as slide preparation, processing, and evaluation of mammary gland whole mounts.

[i] Weights of target organs and organs with gross lesions shall be taken only for those organs scheduled for weighing as listed in Appendix 6. Unless otherwise stated, fix additional tissues in NBF and stain with H&E. If the eye is a target organ or has a gross lesion, it shall be fixed in Modified Davidson's. When non-protocol specified tissues are collected, collect control tissue for histopathology reference when possible.

3. Details for Necropsy and Handling of Reproductive Organs

a) Males (refer to Table 1 of this Histopathology Section for guidance)

- Examine the testes and the gubernacula and note any abnormalities including size, color, location (i.e., cryptorchid), consistency, shape or absence. If the gubernaculum is >20 mm in length in a rat, it is considered elongated. Confirm the absence of the gonadal cranial suspensory ligaments.

- Examine the epididymides for agenesis (record which segments are missing), abnormal size (small or large), presence of fluid or blood within or around epididymides.

- Examine the other reproductive organs in situ, noting any abnormalities, including but not limited to agenesis (i.e., organ/lobe not present), size, color, consistency, and shape.

- Examine the urinary tract for lesions such as renal agenesis, renal atrophy, hydronephrosis, hydroureter, hematuria, and urolithiasis.

- Left Testis and Epididymis: As soon as possible after euthanasia, remove the left testis and epididymis. Immediately dissect the left epididymis from the left testis and trim away any adipose tissue. Make an incision through the epididymis to isolate the cauda from the remainder of the epididymis. Individually weigh the left testis and cauda epididymis and immediately assess the fluid from the cauda for sperm number and motility (note, the left cauda epididymis and the fluid must be kept at 37°C for accurate assessment of sperm motility). Place up to two aliquots of the residual sample of caudal sperm (after assessment of sperm) in individually labeled microcentrifuge tubes, cap, and freeze at -70/-80°C.

  Weigh the remainder of the left epididymis (corpus and caput together) and freeze all portions of left epididymis (cauda [from which the above samples were taken], corpus, and caput) at -70/-80°C. The frozen epididymal sperm samples are to be sent to the NTP Archives for storage. Note: if the left testis has not descended (i.e., is cryptorchid) or if there are any gross lesions in either the left testis or epididymis, then the right testis and epididymis shall be collected for sperm assessment. If both testes are cryptorchid or if there are gross lesions in both testes and/or epididymides, then both testes/epididymides shall be fixed in modified Davidson's fixative and processed for histopathological analysis as described below (i.e., andrological evaluations will not be performed). If only one testis/epididymis is present (i.e., there is unilateral testicular/epididymal agenesis), the testis/epididymis shall be fixed in modified Davidson's fixative and processed for histolopathologic evaluation as described below (andrological evaluations will not be performed).

- Right Testis and Epididymis: Remove the right testis and epididymis. Dissect the right epididymis from the right testis and trim away excess adipose tissue. Weigh the right epididymis and right testis. Using a 21 gauge needle, shallowly pierce the tunica albuginea at each pole of the right testis prior to placement in fixative. Fix right testis and epididymis in modified Davidson's fixative.

- Remove the ventral and dorsolateral prostate, seminal vesicles with coagulating glands. The ventral and dorsolateral prostates are to be separated and weighed individually.

- The LABC muscle complex, preputial glands, and Cowper's (bulbourethral) glands shall be grossly examined in all animals. These organs shall be collected, weighed

and fixed. If there are suspected or demonstrated signs of anti-androgenic activity such as decreased AGD, retained nipples, increased time to preputial separation or other lesions, malformations or abnormalities consistent with anti-androgenic effects, contact the COTR for approval to examine these organs microscopically.

b) Females

The organs of the genitourinary tract shall be examined during necropsy for any lesion, malformation or abnormality using Table 1 for guidance, with particular attention paid to the following:

- Vagina: e.g., agenesis (partial or complete), patency, fluid
- Uterus: e.g., agenesis (unilateral or bilateral, partial or complete), small, swelling/enlargement, and hydrometrocolpus
- Ovaries: e.g., agenesis (unilateral or bilateral), small, swelling/enlargement, ovarian cysts, visible corpora albicans, or the presence of testicular and epididymal tissue (may not be visible grossly)
- Urinary tract: e.g., renal agenesis, renal hypoplasia/atrophy, hydronephrosis, hydroureter, hematuria, urolithiasis, and ectopic ureter/urethra

The uterus, cervix, vagina, and ovaries shall be removed en masse. The ovaries shall be removed from each uterine horn and weighed individually.

In some studies, corpora lutea counts may be required (refer to study protocol). If required, they must be done prior to placing the ovaries in fixative. The counts shall be performed as quickly as possible to minimize autolysis. For a description of corpora lutea of ovulation and corpora lutea of pregnancy, refer to an appropriate textbook, such as Taylor, P. (1986). *Practical Teratology*. London, Academic Press.

Corpora lutea counts can be performed on rats or mice, but require expertise when performed on mice due to their small size. In mice, the outer membrane of the ovary shall be removed to increase the visibility of the corpora lutea. A dissecting microscope shall be used. Record the total number of corpora lutea on the left and right ovaries.

For bred non-littering females only, examine the uterus for nidation (implantation) scars. If no nidation scars are observed, stain the uterus with potassium ferricyanide solution to reveal implantation sites. Potassium ferricyanide staining and implantation site enumeration shall be performed as quickly as possible and the uterus/cervix/vagina shall be placed in fixative as soon as possible to minimize autolysis. Potassium ferricyanide staining is preferred over ammonium sulfide staining for tissue that will be fixed for slide preparation. Use of ammonium sulfide staining is not compatible with fixation and slide preparation.

- Mount the uterus/cervix/vagina (together) on cardboard prior to fixation in NBF to permit sectioning of the entire structure in a single plane.
- Ovaries shall be fixed in Modified Davidson's fixative.

4. Other Organs/Tissues Collected in Reproduction Studies

Collect organs / tissues that are scheduled for routine weighing and examination. See Table 2. Collect any other tissues specified by the protocol, target organs and organs with gross lesions. Refer to Appendix 6 for instructions on collecting those organs not listed below. (If found, multiple,

representative portions of large or heterogeneous tissue masses including surrounding unaffected tissues must be fixed. Masses less than 0.5 cm diameter may be fixed in their entirety.)

- *Adrenal Glands*: Collect the adrenal glands and remove excess surrounding tissue. Examine the adrenal glands for gross lesions. Immerse in NBF.

- *Liver*[1]: Collect the liver, remove excess surrounding tissue, and weigh the liver. Examine the liver for gross lesions. The gall bladder shall be opened in mice. Immerse in 10% NBF.

- *Kidneys*[1]: Collect the kidneys, remove excess surrounding tissue, and weigh the left and right kidneys separately. Examine the kidneys for gross lesions. The kidneys shall be bisected and the cut surfaces examined (before fixation). The left kidney shall be bisected longitudinally and the right kidney transversely. Immerse in 10% NBF.

- *Thyroid Gland*: Dissect the thyroid gland (left and right lobes with parathyroid glands attached) from the trachea, remove excess tissue, and immerse in 10% NBF. The thyroid gland (with attached parathyroid glands) is to be weighed after fixation.

- *Pituitary*: The pituitary shall be fixed *in situ*. After removing the calvarium, externally examine the brain, remove it (leaving the pituitary in the skull), and place it in 10% NBF if the protocol requires the brain to be saved. The nasal bones shall not be removed. Instill NBF into the nasal cavity by gently inserting a blunt needle attached to a syringe into the nasopharyngeal duct and instilling NBF in the nose until drops appear at the external nares. Place the entire calvarium, with the pituitary, in 10% NBF. The pituitary shall be removed for histological preparation after fixation.

- *Mammary Glands (paraffin embedded histologic specimens)/Mammary Gland Whole Mounts*: When required by the study protocol, mammary gland whole mounts shall be collected. Refer to Appendix 6 for instructions on collecting, processing, mounting (on slides), mammary gland whole mounts. Collection of mammary gland from males and/or females for histopathologic examination (paraffin sections) shall be required in all studies in which mammary gland whole mounts are required and may be required in other studies (refer to the study protocol).

  Mammary glands for paraffin embedding and histopathologic examination shall be collected from the right 4th and 5th mammary gland (4th only in PND 90 and older rats). Mammary glands for histopathologic examination shall be collected in exactly the same manner as those for whole mounts and must include the region deep to the 4th nipple and the lymph node. For paraffin embedding and histopathologic evaluation, these glands must be in the same orientation as the glands prepared for whole mounts (thick side toward the label–refer to Appendix 6). Therefore, care must be taken to identify the orientation of the mammary gland during fixation so that they may be placed in cassettes in the proper orientation for processing and trimming. These mammary glands may be placed in a cassette prior to fixation, but if they are not, the mammary gland tissue shall be placed dermal surface down on a piece of cardboard or index card prior to immersion in 10% NBF to keep the sample flat during fixation.

- *Retained Nipples:* In males with retained nipples, one retained nipple from each

[1] These organs are routinely collected, weighed, and fixed. They are examined histologically on a case-by-case basis, if they are known target organs or have gross lesions. Refer to study protocol for specific instructions regarding collection of tissues.

animal shall be collected for histopathologic examination. For retained nipples, the specimen collected shall be centered on the nipple and shall include the adjacent skin and underlying fat pad. If mammary gland is collected for histopathological evaluation from males that have retained nipples, the retained nipple shall be kept in a separate labeled cassette throughout the study.

Following necropsy, the carcasses may be discarded unless specified otherwise by the COTR. In this case, carcasses shall be placed in a properly labeled container and fixed in 10% NBF; saved carcasses shall be discarded only after the pathology evaluation is complete and it is confirmed that no lesions were missed at necropsy (by NTP Pathology peer review). Disposal of the carcasses shall require the approval of the COTR for the relevant NTP contract. In some cases the pelvic portion of the carcass may be saved in reproductive studies.

5. Tissues Collected for RNA, DNA or Protein Assays

   If tissues for RNA, DNA or protein assays are to be collected and frozen, use the instructions in Appendix 6 as well as the SOW.

C. TISSUE FIXATION, TRIMMING AND STAINING

1. Trimming, processing, and embedding of tissues as soon as possible after death is preferred for optimal preservation and possible utilization of tissues for immunohistochemical or other analyses. Tissues in NBF shall be trimmed within a period not less than 24 hours nor greater than 90 days from the day of necropsy for all animals

2. Tissue trimming shall be supervised by the assigned pathologist or a qualified designee, although their continued presence is not required. The necropsy data for each animal shall be available for the technician at the time of tissue trimming. Any additional gross observations shall be recorded during the trimming procedure.

3. All organs/tissues required by the protocol for reproductive studies, all gross lesions, and all known target organs (identified in the study protocol) from all necropsied animals in all dose groups shall be trimmed, processed, and embedded in paraffin, and sectioned at 4-6 μm. The sections shall be mounted on slides and stained with H&E or Hematoxylin/Periodic Acid Shiff's (H/PAS) (see Table 3). (The tissues from the high dose and control groups shall be examined initially. If any treatment-related or unusual lesions are identified in the high dose group, the tissues in which the lesions were identified and any related tissues shall be examined in all dose groups. This applies to all generations, both sexes, and all species, except in some cases for F0 animals for which there was no prenatal treatment.)

4. Tissues fixed in Modified Davidson's fixative: The right testis and epididymis must be fixed in modified Davidson's fixative for 24 hours then either processed and embedded immediately or transferred to 70% ethanol for a maximum of 72 hours after which time they must be processed and embedded (i.e., these tissues must be processed and embedded within 3 days after 24 hours of fixation).

   Other tissues that are fixed in modified Davidson's fixative are fixed for 24 hours, after which they may be immediately processed and embedded, transferred to 70% ethanol and be processed and embedded within 3 days, or transferred to NBF and be processed and embedded within 90 days.

   After removal from modified Davidson's fixative, tissues must be thoroughly rinsed before further processing.

5. Tissues shall be placed in blocks according to an NTP approved scheme in a consistent

manner so that the same tissues are in the same numbered blocks for all animals.

Table 3. Fixation and Staining of Tissues in Reproduction Studies

Organ[a]	Fixative	Stain
Adrenal Gland	NBF	H&E
Liver[b]	NBF	H&E
Kidneys[b]	NBF	H&E
Thyroid Gland	NBF	H&E
Right Testis	Modified Davidson's[c]	H/PAS
Right Epididymis	Modified Davidson's[c]	H/PAS
Dorsolateral Prostate	Modified Davidson's	H&E
Ventral Prostate	Modified Davidson's	H&E
Seminal Vesicles and Coagulating Glands (together)	Modified Davidson's	H&E
Paired Cowper's (Bulbourethral) Glands[d]	Modified Davidson's	H&E
Preputial Glands[d]	NBF	H&E
Levator Ani Bulbocavernosus (LABC) Muscle Complex[d]	NBF	H&E
Right Ovary	Modified Davidson's	H&E
Left Ovary	Modified Davidson's	H&E
Uterus/Cervix/Vagina[e]	NBF	H&E
Retained Nipples[f]	NBF	H&E
Mammary Glands[g]	See Appendix 6	
Pituitary	NBF	H&E
Known Target Organs	NBF[h]	H&E
Gross Lesions	NBF[h]	H&E

[a] Additional organs or tissues may be collected in some studies. In some studies, organs or tissues that are expected to be affected during development may be excluded from examination in the F0 generation. Refer to the study protocol for specific instructions regarding the tissues to be collected. Refer to Appendix 6 for instructions on collecting non-reproductive organs and on trimming non-reproductive organs not listed below. Unless otherwise stated, additional tissues will be fixed in NBF and stained with H&E.

[b] For dose range-finding and standard reproductive and developmental toxicity studies, these organs are to be weighed, examined, and fixed only. They are examined histologically on a case-by-case basis, if they are known target organs or have gross lesions. For histological examination, the organs shall be trimmed, processed to block, cut at 5 microns, and stained with H&E.

[c] Refer to text for processing of right testes and epididymides collected for histopathology.

[d] The Cowper's (bulbourethral) glands, preputial glands and levator ani bulbocavernosus (LABC) muscle complex shall be grossly examined in all necropsied males. However, they shall be collected, fixed, and examined histologically only if there are other lesions, malformations, or abnormalities consistent with anti-androgenic activity.

[e] The uterus/cervix/vagina of each adult female shall be mounted on cardboard prior to placing in the fixative.

[f] One retained nipple from each male with retained nipples shall be collected for histological evaluation in all studies.

[g] Mammary gland whole mounts from males and females may be required in some studies (see study protocol). Refer to Appendix 6 for instructions on collecting, processing, mounting (on slides), and evaluating mammary gland whole mounts.

[h] If the eye is a target organ or has a gross lesion, it shall be fixed in Modified Davidson's fixative rather than NBF.

NBF = 10% neutral buffered formalin, H&E = Hematoxylin and Eosin stain, H/PAS = Hematoxylin/Periodic Acid-Schiff's stain

6. Specific methods for tissue trimming are as follows:

   a. Male Reproductive Organs

      1) *Right Testis*: A single transverse section at the midpoint of the right testis that includes the rete testis is required.

2) *Right Epididymis*: Bisect the right epididymis along the mid-sagittal plane so the head, body, and tail are included in the section. The sample can be cut at the midpoint and embedded in two pieces to allow convenient placement on the slide.

3) *Prostate*: Make mid-transverse sections (approximately 3 mm in thickness) of the dorsolateral lobe and ventral lobe of the prostate (these lobes were separated for weighing prior to fixation). Tissues less than 5 mm in their largest dimension may be embedded whole.

4) *Seminal Vesicle and Coagulating Gland*: Make a mid-transverse section (approx. 3 mm in thickness) of seminal vesicle and coagulating gland bilaterally. The cutting line should be about midway between the anterior and posterior ends. If these tissues are less than 5 mm in their largest dimension, they may be embedded intact.

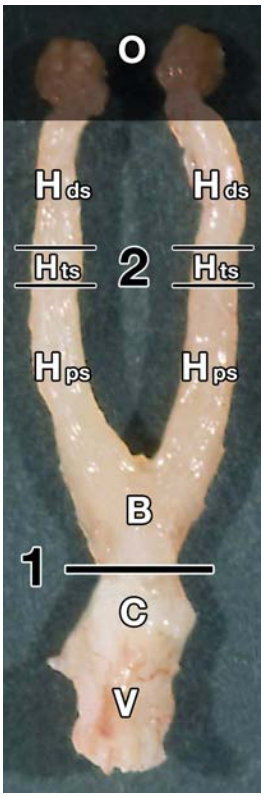
5) *Levator Ani Bulbocavernosus Muscle Group*: Make a mid-transverse section (approx. 5 mm thickness) of the levator ani bulbocavernosus muscle group. If these tissues are less than 5 mm in their largest dimension, they may be embedded intact.

6) *Preputial Glands*: Embed flat so that a longitudinal section is obtained.

b. Female Reproductive Organs

1) *Ovaries*: The ovaries shall be embedded whole such that they may be sectioned parallel to the long axis. Collect the first full-face section at approximately one-third of the way into the ovary. In some cases (see study protocol), the residual ovarian tissue shall be sectioned for ovarian follicle counts (see Appendix 6).

2) *Uterus/Cervix/Vagina*: *Dams*: Trim the cervix and vagina free immediately posterior to the uterine body. Bisect the uterine horns at their midpoint and take one transverse section from the midpoint of each horn. Embed the cervix and vagina (as one piece) and the two transverse uterine sections in a single paraffin block such that a sagittal section of the cervix/vagina and transverse sections of the uterine horns shall be presented for microscopic evaluation. Embed the uterine body with attached portions of uterine horn and the 2 free portions of uterine horn in a 2nd block such that sagittal sections of each tissue shall be presented for microscopic evaluation (see below).



Normal Uterus / Cervix / Vagina

Legend
O = Ovaries
H_{ds} = Uterine horn, distal segment
H_{ts} = Uterine horn, transverse section
H_{ps} = Uterine horn, proximal segment
B = Uterine body
C = Cervix
V = Vagina

Black Lines identified by numbers 1 (one line) and 2 (two lines) indicate the locations where the uterus is to be cut during trimming.

c. Other Organs (See also Appendix 6)

1) *Adrenal Glands*: Sections of the adrenal glands shall include the cortex and medulla.

2) *Liver*[2]: Liver shall be free of adjacent tissues and trimmed to allow the largest cross-section surface area possible for microscopic examination. Two sections of normal liver including sections through the left and median lobes shall be prepared. The sections shall be transverse sections taken midway along the greatest dimension. That of the median lobe shall be taken about 0.5 cm right and lateral to the fissure. If the sections are greater than 2.5 cm in length, one end may be trimmed slightly. In mice, the section of gall bladder must be taken separately and apart from the two liver sections. In addition to these sections, a section shall be taken through any lesion not

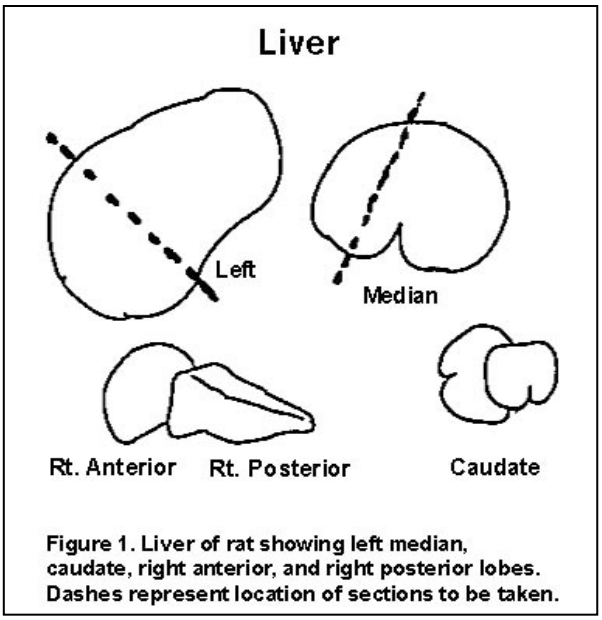


Figure 1. Liver of rat showing left median, caudate, right anterior, and right posterior lobes. Dashes represent location of sections to be taken.

[2] These organs are routinely collected, weighed, and fixed. They are examined histologically on a case-by-case basis, if they are known target organs or have gross lesions. Refer to study protocol for specific instructions regarding collection of tissues.

included in the standard sections. Adjacent normal tissue shall be included along with any lesion. If there are nodular lesions (e.g., tumors), one section of each nodule shall be prepared up to five for each organ. The five largest nodules shall be sectioned if there are more than five.

3) *Kidneys*[3]: Mid-longitudinal section (left kidney) and cross section (right kidney) through the entire cortex, pelvis, and medulla of each kidney shall be submitted.

4) *Thyroid Gland*: After weighing the fixed thyroid gland, a cross section through both lobes of the thyroid, including the parathyroid glands, shall be submitted. If the thyroid gland is less than 0.5 cm in one dimension, it may be processed and embedded whole, but the histopathologically examined section must contain the parathyroid glands.

5) *Mammary Glands (paraffin embedded histologic specimens) / Retained Nipples*: For mammary glands collected for histopathologic examination (right 4th and 5th glands prior to PND 90, 4th only in PND 90 rats), the trimmed sections MUST include the region deep to the 4th nipple and the lymph node. These glands shall be sectioned in the dorsoventral plane (i.e., parallel to the animal's body). The tissue sections must be mounted on slides in the same orientation as the glands for whole mounts (thick side toward the label–refer to Appendix 6). Therefore, care must be taken to identify the orientation of the gland in the cassette and to mount the tissue sections on the slides in the correct orientation. For retained nipples in males, a cross section of the sample containing the nipple and adjacent skin shall be made such that the nipple is in the center of the section and shall include the subjacent mammary fat pad, unless the fat pad is collected for mammary gland evaluation (histopathologic or whole mount).

6) *Pituitary*: Following fixation, the pituitary shall be carefully removed from the calvarium and a coronal section shall be submitted. If the pituitary is less than 0.5 cm in one dimension, it may be processed and embedded whole.

7. Tissues must be trimmed to a maximum thickness of 0.5 cm for processing and embedding. Small (less than 0.5 cm) organs or lesions may be processed and embedded intact. One cross section shall be prepared from each right testis (1 section), seminal vesicle with attached coagulating gland (2), paired Cowper's (bulbourethral) gland (1), preputial glands (2), levator ani bulbocavernosus muscle group (2), dorsolateral prostate (1), ventral prostate (1), ovary (2), uterine horn (2), thyroid gland (2 lobes per animal), pituitary (1), left liver lobe (1)[3], median liver lobe (1)[3], right kidney (1)[3], retained nipple if present (1), and mammary gland if required (1). One longitudinal section shall be prepared from each right epididymis (1), left kidney (1)[3], cervix/vagina (1), uterus (in 3 pieces – one uterine body with attached proximal portions of uterine horns and two free portions of distal uterine horn.

8. All residual tissues from all animals shall be double bagged in NBF following trimming. The animal identification label shall be included with the tissues for all study animals.

[3] These organs are routinely collected, weighed, and fixed. They are examined histologically on a case-by-case basis, if they are known target organs or have gross lesions. Refer to study protocol for specific instructions regarding collection of tissues.

D. HISTOLOGY

1. A unique histology number shall be assigned to each animal that is to receive histologic work-up. At the time of assignment, this number shall be entered in a permanent log and cross-referenced to the animal identification number (if different).

    a. This histology number shall appear on the label placed on the tissue block, on the slide, and on the label between the two bags containing the wet tissues.

    b. All slides (including tissue slides, mammary gland whole mounts, cover-slipped vaginal cytology slides, cover-slipped semen evaluation slides, cover-slipped blood smear slides, reticulocyte preparations and bone marrow preparations, if required by the protocol) shall be labeled using the slide label format presented below. Each block and slide shall be sub-numbered from 1 to n to specifically identify each block/slide for each animal.

    c. The label on the paraffin tissue block and the label between the tissue bags shall have the NTP designated letter code (acronym) for the laboratory preceding the histology number.

2. All trimmed tissues shall be processed (dehydrated and infiltrated with paraffin) using appropriate chemicals, by an automatic tissue processor, sectioned, and stained as indicated in the following sections.

3. After tissues are processed, they shall be embedded in paraffin blocks.

4. Tissues shall be cut 4-6 microns in thickness. Blocks shall be resealed, either by using a warm spatula to melt the surface wax, or by dipping the block in melted paraffin wax. Blocks shall be clearly identified with the designated letter code, plus the laboratory's histology number and sub-numbered from 1 through n to indicate the block number for that animal.

5. Tissue slides shall be stained with hematoxylin and eosin or hematoxylin/periodic acid-Schiff's (see Table 3), after which they shall be cover-slipped with glass cover slips. Each slide shall be permanently paper labeled or labeled using an approved alternate method.

6. Mammary gland whole mounts shall be prepared according to procedures in Appendix 6.

7. All slides shall be subject to quality assessment before microscopic evaluation.

8. Slides shall be compared to the blocks (slide-block match-up) to ensure that all embedded tissues are represented on the slide and that the slide number matches the block number.

9. A Histology Processing Record, electronic or hand-written, shall be completed for each animal for which histology slides are prepared, and shall be submitted to the NTP with the individual animal's necropsy record (IANR). The Histology Processing Record shall include, but is not limited to, the following information:

    a. Header information to include test article, histology accession number, species, dose group, generation, and sex.
    b. List of tissues trimmed, number of cassettes prepared, and verification by trimming technician with initials and date.
    c. List of tissues embedded, number of paraffin blocks prepared, and verification by embedding technician with initials and date.
    d. Number of blocks sectioned, number of slides prepared, and verification by microtomy technician with initials and date.
    e. Number of slides stained and cover-slipped with verification by technician with initials and date.

f. Number of slides checked out during quality control procedure and verification by technician with initials and date.
g. Number of block re-cuts and/or wet tissue re-cuts and verification by technician with initials and date.
h. Notes documenting deviations from protocol, missing tissues, missing gross lesions, problems, and/or comments.
i. Signature of histology laboratory supervisor indicating review and approval of Histology Processing Record.

Format for Slide Labels

Slide labels shall have 4 printed lines with the following information on each line:

Line 1: Laboratory acronym / Pathology subcontractor acronym (if appropriate) / NTP [Acronyms will be supplied by the COTR]
Line 2: Study number – Test number (supplied by COTR)
Line 3: Treatment/dose group and sex designations and individual animal number – Generation and litter designations
Line 4: Histology number – Slide number (the lab performing the histology assigns each animal a histology number which is put on each block)

Treatment/Dose Group, Sex, Generation, and Litter Designations

The treatment/dose group designation will consist of a single letter and will be immediately followed by M for male or F for female, which will be immediately followed by the animal number. The letter designations for treatment/dose groups are as follows:

Dose-Range Finding or Other Short-Term Studies

X = Untreated Control
Y = Vehicle Control
A = Low Dose Group
B = Low medium Dose Group
C = Medium Dose Group
D = Medium High Dose Group
E = High Dose Group
F = High Dose Group (study with 6 treated groups)
G = High Dose Group (study with 7 treated groups)
P = Positive Control

Standard RACB, MOG or Other Long-Term Studies

U = Untreated Control
V = Vehicle Control
L = Low Dose Group
I = Intermediate Dose Group (studies with 4 treated groups)
J = Low Intermediate Dose Group (study with 6 treated groups)
K = Low Intermediate Dose Group (study with 8 treated groups)
M = Medium Dose Group
N = High Intermediate Dose Group (study with 5 treated groups)
O = High Intermediate Dose Group (study with 7 treated groups)
H = High Dose Group
P = Positive Control

The generation and litter designations shall be separated from the treatment/dose group and sex designations by a dash. The generation shall be identified by the letter F followed by the generation number: 0 = original parental generation, 1 = Offspring of F0 generation, 2 = Offspring of F1 generation, 3 = Offspring of F2 generation, etc. The litter will be identified by a lower case letter following the generation designation: a = 1st litter, b = 2nd litter, c = 3rd litter, etc.

Examples:

Dose-Range Finding Study:	AF0011 – F0 Female #11 from the low dose F0 generation
Dose-Range Finding Study:	BM0312 – F1a Male #312 from the 1st litter of the low medium dose F1 generation
Standard RACB Study:	MM2732 – F1c Male #2732 from the 3rd litter of the medium dose F1 generation
Standard RACB Study:	HF3989 – F2b Female #3989 from the 2nd litter of the high dose F2 generation

Sample Slide Label:

RTI/EPL/NTP
05921-02
VF1355–F1c
881750-9

For Mammary gland whole mounts, there is no block so there is no histology number

E. HISTOPATHOLOGIC EVALUATION

1. One pathologist shall microscopically examine and diagnose all tissues, including controls, for a given study. It is preferable that the same pathologist conduct all histopathologic evaluations for an entire study when possible. If necessary, the histopathologic evaluations for a study may be conducted on the females by one pathologist and on the males by another, but the 2 pathologists shall use similar evaluation criteria (e.g., thresholds or grading schemes) as much as possible.

2. Microscopic evaluation by the pathologist shall be performed on the following:

   a. Standard RACB, MOG and Other Long-Term Studies

      Complete histology (making of stained slides) and histopathologic evaluation on early deaths or moribund sacrifices may be required within 30 days after notification and such action is deemed necessary. The NTP COTR in consultation with the Principal Investigator shall make this decision. Unless the NTP requests otherwise, histology and histopathologic evaluation can be postponed to the end of the study. **It must be noted, however, that the testes and epididymides must be trimmed no later than 96 hours post necropsy for all animals regardless of the date of death.**

      Unless otherwise instructed, a complete histopathologic evaluation for reproductive and developmental toxicity studies, inclusive of gross lesions, shall be done on all animals in the control and high dose groups. **Treatment-related lesions/target organs shall be identified and these organs (as well as related organs/tissues) shall be examined in all dose groups.**

   b. Range Finding Studies and Other Short-Term Studies

      Unless otherwise instructed, there will be no histopathologic examination of tissues in reproductive dose range finding studies.

For other short term studies, the test-article specific protocol shall specify any histopathology to be conducted.

3. Complete histopathologic evaluation for reproductive studies is defined as histologic evaluation of the tissues listed below:

Table 4. Tissues for Complete Histopathologic Evaluation for Reproductive Studies

Paired Adrenal Glands	Levator Ani Bulbocavernosus (LABC) Muscle Complex**
Liver (left and median lobes)*	
Kidneys*	Ovaries
Thyroid Gland	Uterus / Cervix / Vagina
Right Testis	Pituitary
Right Epididymis	Retained Nipples (if collected)
Dorsolateral Prostate	Mammary Gland (tissue section, if collected)
Ventral Prostate	
Seminal Vesicles Coagulating Glands	Mammary Gland Whole Mount (if collected)
Paired Cowper's (Bulbourethral) Glands**	Gross Lesions
	Known Target Organs
Preputial Glands**	

* These organs are routinely collected, weighed, and fixed. They are examined histologically on a case-by-case basis, if they are known target organs or have gross lesions. Refer to study protocol for specific instructions.

** The Cowper's (bulbourethral) glands, preputial glands and levator ani bulbocavernosus (LABC) muscle complex shall be grossly examined in all necropsied animals. However, they shall be collected, fixed, and examined histologically only if there are other lesions, malformations, or abnormalities consistent with anti-androgenic activity.

4. The testes shall be evaluated with an awareness of the tubular stages to facilitate a detailed analysis; however, "staging" of the testes need not be performed unless directed by the COTR.
5. Though the seminal vesicles and coagulating glands are collected together, are kept together throughout histologic processing, and are adjacent to each other on the H&E slides, they are to be treated as separate organs during histopathological evaluation and data entry.

F. RECORDING OF HISTOPATHOLOGY RESULTS

All pathological findings for each animal shall be entered into a computerized data management system to capture raw data generated during RACB, MOG, and other studies with reproductive components.

1. The electronic Individual Animal Necropsy Record (IANR) shall be used to record necropsy observations. Descriptive narratives at necropsy shall be provided for all animals. The number as well as description of tissue lesions shall be included (see Appendix 4 for paper version that can be used if electronic is not available).

    a. The IANR shall be used to record the findings of the person performing the necropsy. At the completion of the necropsy, the IANRs shall be signed and dated by the necropsy prosector and the attending pathologist.

    b. IANRs containing necropsy descriptions shall be available to the technician during the trimming process. Any additional gross observations shall be recorded during the trimming procedure, with a separate signature and date for comments added by the trimming technician.

2. The computer terminal shall be used to record all microscopic findings in Reproduction studies.

    a. Use the NTP terminology in the Pathology Code Tables (PCT) supplied by the NTP or modified terminology approved by NTP.

    b. Designated lesions shall be graded using a four-grade system of minimal, mild, moderate, and marked. The PCT shall be used for NTP approved nomenclature.

    c. All gross abnormalities shall be correlated with a microscopic evaluation where applicable.

G. QUALITY CONTROL OF PATHOLOGY ACTIVITIES AND DATA

Quality control of pathology activities and data shall include, but is not limited to, the following procedures:

1. Histology and Histotechnique

    Before slides are given to the pathologist for evaluation, **all** slides shall be examined to ensure that full face sections of the required tissues are present on each slide, that staining of the tissue is optimum, and that the tissue sections have a minimum of artifacts such as folds, knife marks, air bubbles, chatter, shrinkage, etc. Histology records for all animals shall be audited to ensure that all protocol-required tissues and gross lesions have been sectioned or otherwise accounted for, and that sections and slides have been prepared according to NTP guidelines.

2. Residual Wet Tissues

    After all slides have been prepared (e.g. all study protocol required tissues including gross lesions have been trimmed, embedded, sectioned, and stained), the residual wet tissues shall be reviewed for the presence of untrimmed lesions and for animal/carcass identification. A ten percent (10%) random sample of animals of each treatment group shall be examined according to the following guidelines:

    a. All residual tissues from animals in the random samples shall be examined by a pathologist or a histology technician experienced in tissue trimming, for untrimmed lesions. If **any** are found, they shall be confirmed by a pathologist and then residual tissues from all animals of that sex, and generation must be examined for untrimmed lesions.

    b. NOTE: In some organs, such as the liver of rats with mononuclear cell leukemia, the determination that small nodules may be tumors requires considerable scientific judgment and experience. It is imperative that over-sampling and biased sampling of organs does not occur as a result of poor judgment in making these determinations. That is why a pathologist must confirm the presence of untrimmed potential lesions before animals are sampled or additional histologic sections are performed. All potential lesions found as a result of this quality control review shall be trimmed and sections prepared for microscopic examination by the designated study pathologist.

    c. The residual tissues from all animals in the random sample shall be examined for verification of animal/ carcass identification. The identifying markers (tails or other) shall be compared to the bag identification label. If any discrepancy exists, all animals of all treatment groups including controls of that particular sex and species are to be examined. Discrepancies shall be reported to the NTP COTR for that study and an attempt shall be

made to resolve the discrepancies, but bags shall not be relabeled.

3. The completed IANR forms, paper or electronic equivalents, for all animals shall be reviewed for thoroughness of completion, documentation consistency, conformance to NTP Specifications, and for correlation of gross observations with microscopic diagnoses and agreement with the computerized database. If any discrepancies exist, the IANRs shall be returned to the study pathologist or other appropriate personnel for correction of problems prior to auditing by the laboratory QA Unit and subsequent submission to the NTP.

4. If in rare circumstances, the computer entry of histopathology data is performed from a written worksheet, the worksheet shall be retained within the study records and confirmation of its accurate transcription shall be documented on the worksheet and in the computerized data management system. If computer entry of histopathology is performed from an audio recording, all entries must be confirmed by a second person (the second person can be the study pathologist). A record of the primary entry shall be retained and confirmation of its accurate transcription shall be documented with the IANR in the computerized data management system.

5. The histopathology reports shall be reviewed within the laboratory to confirm the correct header information, the correct selection of protocol-required tissues, and the pathology data entry.

6. After the study pathologist completes the first evaluation of the slides, the pathologist shall examine the pathology summary tables for positive or negative trends in the incidences of lesions, and for redundant terminology or inappropriately formatted diagnoses. The pathologist shall re-examine the tissues for which there is a significant positive or negative trend from all animals (by sex/generation) to confirm the initial findings. If redundant terminology is present in the pathology tables, then diagnoses must be changed to consolidate the data in an appropriate manner.

H. SUBMISSION OF PATHOLOGY DATA

1. Standard RACB, MOG and other Long-Term Reproduction Studies

   For standard reproductive studies, the pathology submission to the NTP Archives shall consist of: all tissue slides, blocks, wet tissues, mammary gland whole mount slides, cytology slides, semen-evaluation slides, hematology slides (if blood smears or bone marrow smears are prepared), slide inventory, the original IANRs if paper forms were used, the Histology Processing Records, and notification that the pathology evaluation is complete.

2. Short-Term Studies

   For short-term studies (if histopathology is conducted), the pathology submission to the NTP Archives shall consist of all tissues, slides, blocks, and records generated in the conduct of the study. These may include tissue slides, blocks, wet tissues, mammary gland whole mount slides, cytology slides, semen-evaluation slides, hematology slides (if blood smears or bone marrow smears are prepared), slide inventory, Histology Processing Records, IANRs if paper forms were used, and notification that the pathology is complete.

I. SUBMISSION OF HISTOPATHOLOGY MATERIALS (SLIDES, BLOCKS AND WET TISSUES)

1. General Guidelines

   All slides (including tissue slides, mammary gland whole mounts, cover-slipped vaginal cytology slides, cover-slipped semen evaluation slides, cover-slipped blood smear slides,

reticulocyte preparations and bone marrow preparations, if required by the protocol), blocks, and wet tissues from all animals that are used to generate study data shall be retained at the study lab until approval of the final study report. The histopathology materials shall then be organized, packed, marked, and shipped prepaid to the NTP Archives as directed below.

a. Prior to shipping materials, the Inventory of Residual Material must be completed. A separate inventory shall be submitted for the standard (full) and range finding studies. The number of slides and blocks as well as the condition of wet tissues shall be shown on this form.

b. In addition to the separate inventory of residual histopathology materials provided for each standard and range finding study, the scheme or SOP used to identify the animals in each study (including an appropriate figure or diagram) shall be submitted at the time that wet tissues are sent to the NTP Archives.

c. Blocks and slides shall not be shipped on the same day. The preferred procedure is to ship the blocks first followed by the slides.

d. A letter of intent to ship showing type(s) of histopathology material(s), number of boxes of each, and shipping date(s) shall be directed to the NTP Archives with a copy to the NTP COTR for that study and Pathology Data Coordinator at least 7 calendar days in advance of shipment. This is required to aid in tracing lost or misdirected shipments.

2. Wet Tissues

a. For all studies, all residual animal tissues shall be stored at the NTP Archives in NBF. They shall be double-bagged at the trimming station, packed in animal number, treatment group, and generation order by sex, and shipped to the NTP Archives after completion of the study. For range finding studies, wet tissues are to be retained at the laboratory until submission of the standard full study report, at which time the NTP COTR for that study is to be contacted for disposition of these tissues. If no follow-up standard full study will be performed, the decision regarding disposition of the wet tissues shall be made by NTP COTR for that study following completion of the pathology peer review process and approval of the final data.

b. Wet tissues (residual from harvested tissues) shall be stored in two separately sealed plastic bags, each 4.5 mils thick, one inside the other so that there is no leakage, and organized by species, sex, generation, treatment group, and animal number. A permanent ink label (not ballpoint pen) shall be placed between the two bags showing the study number, laboratory, generation, group number, and animal number. A similar label shall be placed on the external surface of the outer bag. All wet tissues (including mouse carcasses, if applicable), shall be shipped to the NTP Archives in compliance with federal, state, and local shipping regulations. Once the bags are organized, they shall be packed in two layers, separated by a piece of cardboard, within double-wall cardboard boxes (350 lb.-test/51ECT) approximately 15" x 18" x 7.5 " with a plastic liner in each box. The boxes shall be marked on one end to show:

Name of contractor Contract Number Experiment number (R #) Animal group/generation number(s) Histology numbers in that box

Per the OSHA Formaldehyde Standard and the OSHA Hazard Communication Standard,

labels are required for materials containing formalin. A label containing appropriate hazard warnings is to be placed inside the box on **each** container with formalin. An MSDS for formalin is required to be sent to the receiver for the initial shipment, and does not have to be included in the box with the wet tissues, but can be sent under separate cover. The MSDS is not required to be sent with each subsequent shipment from a contractor, only when there is a change in the information in the MSDS.

These boxes shall be sealed shut and bound with filament tape, and shipped promptly to the NTP Archives (in compliance with federal, state, and local shipping regulations) following submission of the final study report or when otherwise specified. Special handling procedures may be required in extreme weather conditions.

3. Blocks shall be resealed by dipping in melted paraffin or with a warm spatula and organized by histology number. Blocks shall be labeled or permanently marked with a laboratory's letter code and the histology number. When histopathology is complete and the residual material, including blocks and slides prepared during range finding and standard study evaluations, are to be prepared for shipment to the NTP Archives, blocks shall be placed in animal order by generation and treatment group into single-wall cardboard boxes the size of approximately eighty blocks. Rows of blocks shall be separated by dividers, in case of partial boxes, spacers will be used to maintain the order of the blocks, and then these smaller boxes (7.5" x 9" x 1.75") shall be taped, and placed into double-wall cardboard containers (350 lb.-test/51ECT) approximately 15" x 18" x 7.5". All boxes shall be marked on one end to show the same information as indicated for wet tissues. Shipping cartons shall be sealed and bound with filament tape for shipment. Special handling procedures may be required in extreme weather conditions.

4. All slides (tissue slides, mammary gland whole mounts, cover-slipped blood smears, cover-slipped cytology slides, cover-slipped semen-evaluation slides, etc. from all phases of the studies) shall be organized by species, sex, generation, treatment group, and animal number, and sent to the NTP Archives when specified or upon completion of the study and submission of the final report. Unstained and uncover-slipped blood smears shall not be shipped to the NTP Archives.

    a. For shipment, the slides shall be placed in plastic slide boxes with "bubble pack" and taped shut. These plastic slide boxes shall be placed in double-walled cardboard boxes (350 lb.-test/51ECT) 15" x 18" x 7.5", separated by abundant packing material, for shipment to the NTP Archives. An Inventory Listing shall accompany the shipment. Slides sent separately in the slide set will be counted as present in the inventory. A copy of the slide-set inventory shall accompany the major inventory document.

    b. Each plastic shipping box shall be marked to show the phase of the study, the treatment, the generation, group numbers, animal numbers and the name of the study laboratory.

    c. Each cardboard box shall contain a packing list identifying the name of the contractor, the number of slide boxes, and the cross-reference information (e.g., animal identification numbers, histology numbers, and study numbers), which will allow complete identification of the contents.

5. Supplies for shipment of residual material to the NTP Archives shall be procured by each testing laboratory.

J. RELEASE OF SLIDES

1. Histologic slides prepared routinely to support these studies shall not leave the contractor's facility without the specific permission of the NTP. If it is necessary to remove slides to obtain

assistance in their interpretation, an inventory sheet shall be prepared and placed in the suspense file until these slides are returned to the contractor's slide file.

2. If it is desired to use sample tissues from these studies for workshops or other purposes where the slides would have to leave the facility, the study laboratory shall first obtain permission from the NTP COTR for that study. After permission is obtained the laboratory shall prepare a separate set of slides and label them in the prescribed manner adding the words "Study Set". Since slides such as these are not used to make diagnoses, the slides shall not be shipped to the NTP Archives upon completion of the study. The NTP COTR and the Head of the NTP Pathology Group shall determine what is to be done with these slides.

K. REFERENCES

Fixation of Testes and Eyes Using a Modified Davidson's Fluid: Comparison with Bouin's Fluid and Conventional Davidson's Fluid. Latendresse, JR, AR Warbritton, H Jonassen and DM Creasy (2002) Toxicol Pathol 30:4, p 524-533.

Specifications for the Conduct of Studies to Evaluate the Toxic and Carcinogenic Potential of Chemical, Biological and Physical Agents in Laboratory Animals for the National Toxicology Program (NTP), 2011.

VIII. CAESAREAN-SECTION AND COLLECTION OF FETAL DATA

Dams shall be euthanized before expected parturition (the morning of GD 21 for rats, GD 18 for mice and GD 30 for rabbits), or as defined in the test article-specific NTP Protocol Outline. Rats and mice shall be euthanized using carbon dioxide inhalation, and rabbits with euthanasia solution administered i.v.

The uterus and ovaries shall be excised, and the number of corpora lutea in each ovary counted and recorded. The uterus of animals that do not appear pregnant shall be examined for nidations (implantation sites) by staining with 0.5% ammonium sulfide (or other suitable agent such as Prussian blue). The weight of the gravid uterus shall be recorded.

The fetuses shall be removed from the uterus and their membranes as soon as possible after the dam is euthanized. The number and location of each implantation shall be recorded. The status of each implant site (live or dead fetus, early or late resorption) shall be recorded. The number of implantations shall be compared to the number of corpora lutea to determine pre-implantation loss.

- An early resorption is a conceptus characterized by a grossly necrotic mass that has no recognizable fetal form; nidation sites ("pregnant by stain") are also considered to be early resorptions.
- A late resorption is characterized by a grossly necrotic but recognizable fetal form with placental remnants visible.
- A live fetus is pink in color and responds to stimulation.
- A dead fetus is often pale to tan in color and is not responsive to stimulation but does not demonstrate marked autolysis. Fetuses with marked autolysis are considered to be late resorptions.

Fetuses shall be separated from the placenta by cutting the umbilical cord, and any membranes removed. The fetuses shall be blotted dry, individually identified, and individually weighed. Placentas shall be examined for abnormalities in appearance.

Each fetus shall receive an examination as described below. Examination findings shall be recorded in accordance with the NTP computer data system library (or other NTP-approved process).

Rodent and rabbit dose range-finding studies:

- Fetal weights shall be obtained and external examinations shall be conducted
- The external examination shall include examination of all body surfaces (including the offices) and appendages. The mouth shall be opened and examined for cleft palate.
- The gender of each fetus shall be determined, by inspection of ano-genital distance (AGD) for rodents and internally for rabbits (internal confirmation for rodents when it is in doubt).

Definitive rodent and rabbit studies:

- All fetuses shall be subjected to external, visceral and skeletal examinations.
- Visceral and skeletal examinations shall be conducted under a dissecting microscope
- The sex of each fetus shall be confirmed (or in the case of rabbits determined) by internal examination of the gonads.
- Approximately 50% of each litter shall have the head removed just below the exoccipitals leaving the cervical region intact, Bouin's-fixed (rodents), and subjected to examination by serial razor blade sectioning. The remaining carcass shall be processed for skeletal examination (along with the other half of carcasses). Fetuses shall be tagged in a manner so that they can be identified throughout processing, reading and archiving.
- All skeletons shall be double-stained with Alcian blue (0.05% in 5% acetic acid) and alizarin red (0.005% (w/v) in 1% potassium hydroxide) and stored in glycerol: ethanol (1:1).[4] Other methodology may be approved by the COTR.

[4] Alcian Blue/Alizarin Red Staining of Cartilage and Bone in Mouse ,Dmitry Ovchinnikov Cold Spring Harb Protoc; 2009; doi:10.1101/pdb.prot5170

The external examination shall include:

- Fetal weight, external examination of all body surfaces (including the offices) and appendages. The mouth shall be opened and examined for cleft palate.

The visceral examination shall include:

- Examination and evaluation of the abdominal viscera including:
  - The organs of the digestive system (intestines, stomach, pancreas, spleen, liver [and gall bladder]), urinary system (kidneys, ureters, urinary bladder), adrenals, and reproductive system (for the female: ovaries and uterus or for the male: testes, epididymides). Kidneys shall be sectioned at the hilus and the renal papilla examined.
- The following thoracic viscera shall be examined and evaluated:
  - The trachea, esophagus, thymus, lungs, diaphragm, and greater vessels of the heart (right and left subclavians and carotids, innominate, pulmonary arch, pulmonary artery, vena cava, aortic arch, aorta, and the ductus arteriosus).
- The following aspects of the heart and vessels shall be examined and evaluated:
  - The shape and position shall be examined and any abnormalities recorded.
  - The aorta, pulmonary artery, descending aorta, innominate (brachiocephalic), subclavian, common carotid arteries and the ductus arteriosus shall be examined.
  - The internal anatomy of the heart shall be examined including the aortic, tricuspid, mitral and pulmonic valves, and interventricular septum

The skeletal exam shall include:

- Examination of the bones of the skull:
  - premaxillae, maxillae, nasals, frontals, parietals, interparietal, supraoccipital, exoccipitals, zygomatics, squamosals and mandibles.
- Examination of axial skeleton
  - Vertebrae (centra and arches)
  - sternebrae
  - ribs
- Examination of the pectoral girdle and pelvic girdle
  - dorsal scapulae and clavicles
  - ilia, ischia, and pubis
- Examination of the forelimbs and hindlimbs
  - humerus, radius, ulna, carpals, metacarpals, phalanges
  - femur, tibia, fibula, tarsals, metatarsals, phalanges

Fetal head examination[5] shall include:

- A ventrodorsal section (mouth and bisecting both ears) shall be made and the following tissues examined:
  - tongue, palate, upper lips, and lower jaw.
- A frontal section anterior to the eyes shall be made and the following tissues examined:
  - nasal septum, nasal sinuses, and palate.
- A frontal section that bisects the eyes shall be made and the following tissues examined:
  - olfactory lobes of the brain, posterior areas of the nasal septum, nasal sinus (nasopharyngeal cavity), palate, and fetal eye (including optic cup, retina, vitreous chamber, lens, and cornea).
- A frontal section posterior to the eyes (middle of the parietals) shall be made and the cerebral hemispheres (including the 3rd and lateral ventricles) examined.
- Sections shall be retained in a manner in which each fetal head may be reassessed.

---

[5] Thompson, R. F. (1967). Basic Neuroanatomy, Chapter 4, In: *Foundations of Physiological Psychology.* R. F. Thompson (ed.). Harper and Row Publishers, New York, pp. 79-82.

During the fetal examinations, all unusual (notable) observations shall be recorded using an NTP-approved data collection system. The observations shall subsequently be determined to be "malformations" or "variations" based on established criteria and designations (consistent with Makris et al, Terminology of Developmental Abnormalities in Common Laboratory Mammals (v.2) 2009, Birth Defects Research Part B, 86:4). Historical control data are essential (along with concurrent controls) to determine the designation and occurrence of the findings in the context of normal background. In general, malformations are considered to be incompatible with, or severely detrimental to postnatal survival, for example: ventricular septal defect, exencephaly, diaphragmatic hernia. Variations are non-lethal and not considered detrimental to postnatal survival such as reduced ossification due to toxicity-related developmental delay, for example: reduced ossification of fore- and hind paws or caudal vertebrae.

See Section XII – Report Formats for sample reporting tables and calculation of endpoints such as mating, fertility and fecundity indices.

IX. QUALITY ASSURANCE

A. GOOD LABORATORY PRACTICE (GLP) REQUIREMENTS

1. The NTP requires that studies be conducted in compliance with FDA GLP regulations as specified in 21 CFR Part 58 "Good Laboratory Practices for Non-clinical Laboratory Studies." There are areas where NTP has specific requirements that extend the FDA requirements. In these areas, the laboratory is expected to provide NTP with those requirements as described in these Specifications.

2. There are NTP studies for which the EPA GLP Standards are more appropriate than the FDA GLP Regulations. To meet the need for compliance, it may be necessary to conduct NTP studies in compliance with the requirements of EPA as well as FDA. (40 CFR Part 160; 40 CFR Part 792.)

3. The review and revision of Standard Operating Procedures (SOPs) is a continuing process. Along with the protocol, SOPs are considered essential to the successful conduct, documentation, inspection, and auditing of a study. For this reason, all SOPs must be reviewed at least every two years. New or revised SOPs need to be prepared, reviewed and approved by the testing laboratory before they are implemented.

4. A laboratory can expect to have Quality Assurance (QA) monitoring site visits periodically. During the visit, NTP QA staff will evaluate the organization and function of the laboratory's Quality Assurance Unit (QAU). Additional follow-up visits will be made, if needed. A team of two to four QA professionals (support contract and NTP staff) may also visit a laboratory to audit and inspect representative ongoing studies.

B. THE QAU

1. If a laboratory or facility has multiple QAUs, based on the division of operation [e.g. Biology, Chemistry, Pathology, or other disciplines], that QAU serving the basic animal studies shall be responsible for assessing the quality of all aspects of the studies. Whether or not SOPs are prepared specific to the NTP program is to be determined by laboratory management. In either case, SOPs used in connection with each NTP study shall be maintained as NTP records for archiving purposes. These SOPs are subject to review by NTP personnel as to interpretation of what is to be done.

2. The QAU for the laboratory shall develop and maintain specific QA SOPs. These SOPs are to cover QA activities that are repetitive in nature and deal with the independent conduct of inspections, audits, and related activities associated with NTP studies performed by the contract laboratory. An historical file of these SOPs and all revisions thereof, including the dates of such revisions, shall be maintained.

3. The laboratory shall maintain a file of QAU reports (inspections, audits, periodic status) and responses to them in connection with NTP studies. As the sponsor for the studies, NTP management, QA personnel, and the COTR shall have access to the file for review purposes. The confidential, proprietary, and pre-decisional information contained in this file shall not be divulged by NTP reviewers. The file shall not be revealed to any outside parties, including QA support contractors for the NTP or GLP compliance inspectors for the FDA or EPA. When a study is completed, the QAU reports file shall not be part of the study record and shall not be submitted to the NTP Archives.

C. AUDITS AND INSPECTIONS

Phases are to be inspected and resulting data audited according to frequencies based on the nature of the data collected, the extent of quality control (QC) review, and the potential impact of errors. These factors and the monitoring frequency are to be considered and established in concert with the critical staff and the laboratory's management. The schedule of inspections and audits reflects a dynamic process and will be influenced by a variety of factors such as previous findings, follow-up activities, workload and quality issues expressed by management, inspectional findings from outside reviewers, etc. The following sections outline the approach that shall be used for organizing inspections and audits of NTP studies by the laboratory QAU.

1. Prestart activities conducted during phase 1 are to be inspected and data/reports audited. These include, but are not limited to: receipt of test article; storage and handling of test article; purity and identity of test article; method performance evaluation; SOPs; prestart reports for chemistry, toxicokinetics; special proficiency studies, etc.

2. The study protocol and amendments must be reviewed by the laboratory's QAU for compliance with contractual requirements, the NTP Specifications, and GLP regulations. Furthermore, the laboratory's QA officer is required to sign and date the study protocol prior to the initiation of the study to document that the study protocol has been received and reviewed by the laboratory's QAU.

3. Those phases/events of a study that occur only once and include procedures or conditions that can be directly observed are to be inspected at the time they occur and resulting data audited. Examples of such phases include animal quarantine, randomization, and individual identification; release to study, study start; clinical pathology; special study events and; scheduled necropsy; tissue trimming, and slide preparation. If a once-only phase undergoes a formal and documented QC review by technical staff, the QAU may limit its responsibility to inspecting the procedures of the quality control review for that phase.

4. Repetitive or routine procedures that impact the generation, collection, and handling of study data need to be subjected to inspection and audit on a periodic basis for each study. A few examples of such procedures include the control of environmental conditions; dose formulation, analysis, and administration; individual animal weights, feed or water consumption, clinical signs, and survival; animal husbandry practices; necropsy and processing of unscheduled-death animals; and documentation practices and error corrections.

5. All data and statements of fact included in study reports submitted to the NTP are to be audited by the laboratory's QAU, with the exception of the Monthly Progress Reports and preliminary data submissions that are defined in each contract.

X. GENERAL STUDY PROTOCOL OUTLINES

Each test article-specific NTP protocol shall be customized to reflect optional endpoints. This section contains typical protocol outlines/templates for:

A. Reproductive Assessment by Continuous Breeding (RACB) Study,

B. Prenatal Developmental Toxicity Study, and

C. NTP Modified One Generation Study

The design of these study protocol outlines may be varied as needed.

PROTOCOL OUTLINE FOR THE STUDY OF A TEST ARTICLE ADMINISTERED BY (ROUTE) IN HARLAN SPRAGUE DAWLEY RATS USING THE NTP REPRODUCTIVE ASSESSMENT BY CONTINUOUS BREEDING (RACB) DESIGN [NTP Study Number RXXXX]

1. INTRODUCTION:

1.1. Background and Rationale (test article specific)

2. **OBJECTIVE:** To characterize the reproductive toxicity of the Test Article in Harlan Sprague Dawley rats using the NTP RACB design.

The RACB is composed of four interrelated parts: (1) if the acceptable range of dose levels required to avoid general toxicity is unknown, a dose range-finding (DRF) study is conducted; (2) cohabitation of F_0 males and females to produce three (3) F_1 generation litters for assessment, with chemical administered continuously from pre-cohabitation of F_0 animals and weaning of F_1 animals until their necropsy; (3) if an effect on the fertility or fecundity of F_0 animals is seen, a crossover mating of F_0 high-dose exposed males with näive unexposed females; and high-dose exposed females with control males shall be conducted to determine the affected sex(es); and (4) cohabitation of F_1 males and females to produce three (3) F_2 generation litters for assessment, with chemical administered continuously to F_1 animals during cohabitation and to F_2 animals from weaning until their necropsy.

3. TEST ARTICLE

3.1. Stucture, Molecular Formula, Molecular Weight, CAS #, Source

3.2. Characterization: The composition/purity of the bulk material shall be assayed within 30 days prior to beginning, projected middle and within thirty days after euthanizing the last animal on the definitive RACB study. A representative sample from each initial, middle and final formulated material preparation shall be analyzed to confirm concentration in the DRF. For the full study, formulated material shall be analyzed initially and every 10±2 weeks for concentration. If batch sizes are different between the DRF and full study, a new homogeneity study is required.

3.3. **Route of Administration oral Feed or Gavage** *(dose volume e.g. 2 or 5 mL/kg)* or (other)

3.4. Vehicle information (w/ CAS #, if appropriate)

4. TEST ANIMALS

4.1. Species, Strain/Stock and Age: Male and Female Harlan Sprague Dawley Rats (Harlan Laboratories). F_0 female and male rats shall be sexually mature at the start of mating (at least 11 and 13 weeks of age, respectively).

4.2. Diet and Water: Tap water and Feed (NIH-07; from an NTP-approved vendor) to be provided ad libitum.

4.3. Pre-study evaluation: Animals shall be obtained 10-14 days prior to study start and undergo quarantine prior to use. Before study start, 5 males and 5 females shall undergo a health assessment to ensure the health of the animals on study.

4.4. Randomization and Identification: Prior to dosing, F_0 rats shall be randomized into treatment groups by sex using a method to minimize the variance in mean body weight, and uniquely

identified. F_{1c} and F_{2c} pups shall be identified by paw tattoo according to number within litter at PND 1 and, when retained beyond PND 4, uniquely identified at weaning.

4.5. Euthanasia: Discomfort or injury to animals shall be limited, in that if any animal, including pre-weaning F_1 or F_2 offspring, becomes severely debilitated or moribund, it shall be humanely euthanized.

5. PROCEDURES

5.1. Study Design: Dose Range-Finding Study (DRF) *(if required to set dose levels for the RACB study); additional animals may be added for biological sample analysis to determine test article concentrations in dams and pups).*

5.1.1. Caging

Rats shall be housed up to five per cage (depending on cage size) by sex and dose group until they are paired for mating. During the mating cohabitation period, rats shall be housed one male and one female per cage from the same dose group (up to 15 days, or until positive evidence of mating). After evidence of mating, or after 15 days if no evidence of mating, the pairs shall be separated and individually housed until necropsy. Pups shall remain with their dam until necropsy on PND 4 (or PND 21 or 28).

5.1.2. Dose levels and number of rats

Rats shall be randomized as described in **Section 4.4** into 6 groups and continuously exposed via feed containing the ppm indicated below (or dosed water or gavage):

Group	Dose levels *(mg/kg, ppm, etc)*	No. of F_0 males	No. of F_0 females
Control (1)		8	8
2		8	8
3		8	8
4		8	8
5		8	8
6		8	8
	Total F_0	**48**	**48**

5.1.3. Duration of dosing *(and frequency if gavage)*

Males and females shall be dosed daily/continuously for 7 days/week throughout the entire study: pre-cohabitation dosing period (generally 7 days, but may be longer for some test articles), during the cohabitation breeding period, gestation, and until the rats and pups are euthanized on PND 4 (i.e. up to 56 days) (*or longer if specified by the COTR*). PND 0 is defined as the day that all pups within a litter have been delivered. *Note- For gavage studies, dose volume shall be based on the most recent body weight.*

5.1.4. Cohabitation

Male and female rats shall be housed together up to 15 days, or until positive evidence of mating. If after 15 days there is no evidence of mating, then the rats shall be separated and the females monitored for delivery up 23-24 days. Day of positive evidence of mating = GD 0.

5.1.5. In-life endpoints

- Time to mating (pre-coital interval)
- Formal (out of cage) clinical observations once daily (for gavage studies the SOW may specify a specific time frame such as 1-3 hrs post dose); cage side observations, twice daily (before 10AM and after 2PM) including signs of delivery, moribundity and/or death.
- Male body weights twice weekly *(NOTE- twice weekly is defined as every three, then*

4 days). Female body weights, twice weekly at all times during the study except during gestation when they shall be recorded daily; PND 1 and 4. Twice weekly weights are to be reported as well as gestational/post-natal weights for the following intervals: GD 0-3, 3-6, 6-9, 9-12, 12-15, 15-18, 18-21, and 0-21; PND 1-4.

- Male feed consumption twice weekly (unless breeding). Female feed consumption twice weekly prior to mating, and for *(include water consumption- if Test Article is administered in water)* GD 0-3, 3-6, 6-9, 9-12, 12-15, 15-18, 18-21, 0-21, and PND 1-4. Food consumption data shall not be collected during cohabitation.
- Number of live and dead pups delivered by sex and by litter and total on PND 0.
- Number and weight of live pups by sex and by litter and total on PND 1 and 4
- Number of non-pregnant rats

5.1.6. Euthanasia

Males shall be euthanized after the cohabitation period and discarded without examination. Females and pups shall be euthanized on PND 4 (or sufficiently after expected littering date, if no evidence of pregnancy). Rats that produced litters shall be discarded without examination. Rats that do not appear to be pregnant and do not deliver shall be examined for the presence of implants (using potassium ferricyanide or ammonium sulfide) and corpora lutea enumerated.

5.2. Study Design: Full RACB

5.2.1. Caging

Rats shall be housed up to five per sex per cage (depending on cage size) until they are paired for mating. During the mating cohabitation periods, rats shall be housed as pairs of one male and one female per cage from the same dose group (up to 15 days, or until positive evidence of mating). After evidence of mating, or after 15 days if no evidence of mating, the pairs shall be separated and individually housed until cohabited again for production of the next litter or until necropsy. The same male and female are paired each time as "life-long mating pairs". Pups shall remain with their respective dam until termination or weaning. After weaning, juveniles shall be group housed by litter and sex (2-3 per cage as appropriate for their size). Weaning is on PND 21 (or 28 for HSD).

5.2.2. Dose levels and number of animals

Rats shall be randomized as described in **Section 4.4** into 4 groups:

Group	Dose levels[a] (mg/kg, ppm, etc)	No. of F_0 males	No. of F_0 females
Vehicle Control		23	23
Low Dose		23	23
Mid-Dose		23	23
High-Dose		23	23
	Total F0	**92**	**92**

[a] F_{1c} and F_{2c} offspring shall also be directly administered the test article post-weaning at the same dose as their parents.

5.2.3. Duration of dosing

F_0 rats shall be administered control- or dosed-feed ad libitum continuously (or water containing the test article) or dosed daily by gavage- include dose volume [e.g. 2 or 5 mL/kg]), 7 days a week, from the pre-cohabitation dosing period (typically 2 weeks prior to cohabitation for both males and females), throughout breeding and gestation, and until necropsy. F_1 and F_2 offspring shall be exposed via nursing. F_{1c} and F_{2c} shall also be exposed to treatment after weaning, at the same dose as their parents, 7 days a week until euthanasia, or scheduled necropsy.

5.2.4. F_0 mating and $F_{1(a,b,c)}$ production, randomization and disposition

After the last day of the pre-cohabitation dosing interval, F_0 males and females shall be cohabitated (one male and one female as "a life-long mating-pair" per cage). After evidence of mating (or after 15 days if no evidence of mating), male and females shall be separated and females shall be allowed to litter. The same breeding pairs shall undergo re-cohabitation 2 days after their F_{1a} and F_{1b} litters have been euthanized.

F_{1a} and F_{1b} litters shall be euthanized on PND 4, typically with no further examination (however, in some cases, they may be subjected to a gross examination); they do not require a paw tattoo.

(Optional endpoints)
The inguinal mammary glands from ten males/dose level and ten females/dose level randomly selected from the F_{1b} culls on PND 4 (from different litters) shall be collected on PND 4 and whole mounted for subsequent assessment of abnormal mammary gland development. Animals may also be used for collection of biological samples for other evaluations such as test article concentration.

Pups of the F_{1c} litter shall be uniquely identified on PND 1 by paw tattoo. When possible, the F_{1c} litters shall be standardized to up to 11 pups/litter on PND 4 by randomly selecting 2 males and 2 females per litter as future breeders, 3 males and 3 females per litter for PND 91 histopathological evaluations, and 1 male or 1 female from each of 10 litters (if available) having excess males or females, respectively, for preparation of mammary gland whole-mounts (if required). Non-selected F_{1c} pups shall be euthanized on PND 4 as described above. *Note- if it is not possible to determine sex on PND 4 (i.e. apparent skewed sex ratio), culling may need to be delayed until weaning.*

(Optional endpoint)
The inguinal mammary glands from one F_{1c} offspring/sex/litter at the time of vaginal opening (female) or PND 21or 28 (male) shall be collected and whole mounted (up to ten/sex/dose level at the appropriate interval). These animals shall be given a gross examination and tissues with gross lesions (and comparative controls) shall be fixed and retained for subsequent histopathological evaluation.

At the time of weaning two males and two females from each litter shall be randomly assigned (within dose; avoiding sibling pairings) as breeders to produce the next (F_2) generation.

- See note in **Section 7** regarding potential effects on fertility.

5.2.5. In-life endpoints

5.2.5.1. F_0 males

- Formal (out of cage) clinical observations once daily; cage side observations, including, moribundity and/or death, twice daily (before 10AM and after 2PM)
- Cage feed consumption (and water consumption when the test article is administered in the water), twice /week (unless breeding).
- Body weights twice weekly.

5.2.5.2. F_0 dams producing $F_{1a\ and\ b}$ litters

- Formal (out of cage) clinical observations once daily; cage side observations, including, moribundity and/or death, twice daily (before 10AM and after 2PM)
- Female body weights, twice weekly at all times during the study except during gestation when they shall be recorded daily; PND 1 and 4. Twice weekly weights are to be reported as well as gestational/post-natal weights for the

following intervals: GD 0-3, 3-6, 6-9, 9-12, 12-15, 15-18, 18-21, and 0-21; PND 1-4.

- Feed consumption (and water consumption-if administered in the water) twice weekly (when not breeding/pregnant) and GD 0-3, 3-6, 6-9, 9-12, 12-15, 15-18, 18-21, 0-21; PND 1-4
- Time to mating (pre-coital interval)
- Number of pregnant females, number of litters per pair, and duration of pregnancy.

5.2.5.3. $F_{1a \text{ and } b}$ pups

- PND 0: Number of live and dead offspring (by litter, sex, and total)
- PND 1: Number of live and dead offspring (by litter, sex, and total); anogenital distance (AGD) and corresponding pup weight.
- PND 4: Number of live and dead pups, sex, weight (by litter, sex, and total).
- Presence and type of developmental anomalies in the pups (e.g. clinical observations, gross external malformations).

5.2.5.4. F_0 dams producing F_{1c} litters

- Formal (out of cage) observations once daily; cage side observations including signs of delivery, morbidity and/or death, twice daily (before 10AM and after 2PM). Care shall be taken so that dams in the process of delivery are not disturbed.
- Female body weights, twice weekly prior to mating, daily during gestation and PND 1, 4, 7, 10, 13, 16, 19, 21 and 28; at termination. Body weight changes to be reported for the following intervals: GD 0-3, 3-6, 6-9, 9-12, 12-15, 15-18, 18-21, 0-21, PND 1-4, 4-7, 7-10, 10-13, 13-16, 16-19, 19-21; PND 1-16, 1-21, 1-28.
- Feed consumption (and water consumption-if exposure is via the water) twice weekly (when not mated/not pregnant); GD 0-3, 3-6, 6-9, 9-12, 12-15, 15-18, 18-20; PND 1-4, 4-7 7-10, 10-13, 13-16, 16-19, 19-21 and PND 1-16, 1-28.
- Time to mating (pre-coital interval)
- Number of pregnant dams, no.of litters per pair, and duration of pregnancy.

5.2.5.5. F_{1c}offspring

- All endpoints noted above for $F1_{a \text{ and } b}$ pups (including pre-cull PND 4 weights).
- Number and weight of pups on PND 4 (post cull), 7, 10, 13, 16, 19, 21 *(and PND 28)* (by pup, sex and litter). Pup body weight gain changes by sex to be reported for the following intervals 1-4 pre cull, 4 (post cull)-7, 7-10, 10-13, 13-16, 16-19, 19-21, post cull 4-21 or 4-28.
- Males: Presence of areolae on PND 13, testicular descent (PND 14-30), balano-preputial separation (BPS; PND 35 until acquisition; the NTP-COTR shall be consulted if BPS has not occurred by PND 50) and body weight on day of acquisition.
- Females: day of vaginal opening (PND 23 until acquisition; the COTR shall be consulted if VO has not occurred by PND 42) and body weight on day of acquisition and estrous cyclicity (starting at PND 77 $\pm$ 3 days and continuing for 16 consecutive days).
- After weaning: weekly body weights and cage feed consumption *(and water consumption if exposure is via the water).*

5.2.6.F_0 Necropsy

Prior to necropsy of F_0 females, the Contractor shall have conducted vaginal smears for 16 days to determine estrous cyclicity. F_0 animals shall be necropsied as soon as they are no longer needed (i.e., after completion of any crossover mating [see note in **Section**

7 regarding potential effects on fertility], F_1 offspring have been weaned, and vaginal cytology assessment of F_0 females is complete).

5.2.6.1. The following endpoints shall be measured:

- Left cauda epididymal spermatzoa measurements: number, density, motility.
- Left testicular measurements: spermatid head count. Note- in the case of unilateral effects on organ weights, or presence of gross lesions, pathology takes precedence over sperm assessments in selection of right or left testis/ epididymis.
- Organ weights:

Adrenal Glands (paired)	Ventral Prostate
Epididymis, right and left	Ovaries, right and left
Cauda Epididymis, left[a]	Kidneys
Testis, right and left	Liver
Seminal Vesicles (w/ Coagulating Glands)	Thymus
Dorsal Prostate	Thyroid Gland
a: Side processed for sperm analyses	

- Fix and process the following tissues[a] per NTP specifications:

Adrenal Glands	Pituitary
Vagina, cervix, and uterus (together)	Kidneys [c]
Epididymis, right [b]	Liver [c]
Testis, right [b]	Thymus [c]
Seminal Vesicles and Coagulating Glands (together)	Thyroid Gland
Dorso-lateral Prostate	Gross lesions
Ventral Prostate	
Ovaries	
a: All tissues shall be fixed in formalin with the exception of the testes and epididymides which shall be fixed in modified Davidsons b: If the rat exhibits a gross findings on the left, rather than the right, collect, process, and examine the left tissues for histopathology. c: These tissues shall be fixed, but processed and examined histologically on a case-by-case basis, if they are known target organs or have gross lesions; refer to the study protocol.	

Fixed tissues shall be processed to slides (except where noted) and stained with hemotoxylin and eosin. The high dose and control groups shall be examined for histopathological changes. If changes are found, all dose groups shall be examined for these and any related changes as defined by the NTP COTR.

5.2.7.F_{1c} Estrous cyclicity, mating and F_2 assessment:

When male F_{1c} rats reach PND 91 (full sexual maturity) two males and two females shall be randomly selected from each F_{1c} litter to produce non-sibling mating pairs (i.e. up to 46 pairs/ dose group), for the purpose of producing three F_2 litters/pair for assessment. *Note- the endpoints outlined in Section 5.2.5.5 shall be assessed on all non-culled offspring*

Vaginal cytology data shall be collected on the F_{1c} females for at least 16 days prior mating (or euthanasia for females not selected for breeding).

The production and assessment of F_2 litters shall be the same as the F_1 litter production and assessment (See **sections 5.2.4** through **5.2.6**). Once the F_{2c} litter is weaned, the offspring shall be administered the test article until necropsy (PND 28 or 91 as directed by the COTR). If F_{2c} females are maintained until adulthood, vaginal cytology data is to be collected on them for 16 days prior to euthanasia.

After delivery of the F2c litter, the F1c breeding pairs shall be retained for at least 28 days.

5.2.8. F_1 and F_2 Necropsy

5.2.8.1. Non-selected F_{1c} and F_{2c} pups at time of culling (PND 4)

See **Section 5.2.4**

5.2.8.2. F_{1c} Necropsy

- Male pup (one/litter) selected on PND 28; female pup (one/litter) randomly at time of vaginal opening shall be examined as discussed in **Section 5.2.4.**
- F_{1c} females not selected for breeding shall be necropsied after the vaginal cytology assessment of F_{1c} females is complete.
- Bred F_{1c} females shall be necropsied after of their F_2 offspring have been euthanized or weaned.
- F_{1c} males shall be necropsied after separation following the 3rd mating period. Trunk blood shall be collected and sera isolated (target volume 2mL) and frozen for potential hormone (or other potential) analyses as directed by the NTP-COTR. If areolae are noted on male rats at PND 13, the ventral surface of male rats shall be shaved and nipples counted.

- **The following endpoints shall also be measured:**
  Left cauda epididymal spermatozoa measurements: number, density, motility.

- Left testicular measurements: spermatid head count. *Note- in the case of unilateral effects on organ weights, or presence of gross lesions, pathology takes precedence over sperm assessments in selection of right or left testis/ epididymis.*

- Organ weights:

Adrenal glands (paired)	Ventral Prostate
Epididymis, right and left	Ovaries, right and left
Cauda Epididymis, right and left	Kidneys
Testis, right and left	Liver
Seminal Vesicles (w/ Coagulating Glands)	Thymus
Dorso-lateral Prostate	Thyroid Gland
Levator ani bulbocavernosus (LABC)[a]	Preputial glands[a]
a: Only collected and weighed when an antiandrogenic effect is expected (e.g. observed increased incidence of areolae/nipples, delay in BPS)	

- Fix and process the following tissues[a] per NTP specifications:

Adrenal Glands	Pituitary
Vagina, cervix, and uterus (together)	Kidneys [c]
Epididymis, right [b]	Liver [c]
Testis, right [b]	Thymus [c]
Seminal Vesicles and Coagulating Glands (together)	Thyroid Gland
Dorsal Prostate	Gross lesions
Ventral Prostate	Mammary gland/nipples
Ovaries	

a: All tissues shall be fixed in formalin with the exception of the testes and epididymides which shall be fixed in modified Davidsons
b: If the rat exhibits gross findings on the left, rather than the right, collect, process, and examine the left tissues for histopathology
c: These tissues shall be fixed, but processed and examined histologically on a case-by-case basis, if they are known target organs or have gross lesions; refer to the study protocol.

Fixed tissues shall be processed to slides (except where noted) and stained with hemotoxylin and eosin. The high and control groups shall be examined for histopathological changes. If high dose effects are found, all the remaining dose groups shall be examined for these and any related changes as defined by the NTP COTR.

5.2.8.3. F_{2c} Necropsy

If F_{2c} pups are retained and dosing continued after PND 28, these animals shall be necropsied on (or around) PND 91 after vaginal cytology assessment of F_{2c} females is complete. Endpoints described in F_{1c} necropsy (**Section 5.2.8.2**) shall be collected on each animal.

5.2.9. Data Presentation

Mean values and index of variability (i.e. SE, or SD) for all protocol specified parameters shall be provided to the COTR, including (but not limited to):

- Mean body weights, body weight gains, food and water consumption (see **Sections 5.1.5** and **5.2.5**)
- Male and female fecundity and fertility indices
- Mean litter size, mean body weight of live pups (by sex, and combined) and total number live/dead pups.
- Pup survival on PND 1-4, 4-7, 7-14, 14-21, 21-28
- AGD (by sex, nested by litter and co-varied by litter size)
- Mean number of areolae and nipples (by litter)
- Organ weights

Note- Summaries of data shall be provided to the COTR within five days after each of the following:

- End of the DRF study;
- Delivery of the last individual dam for each F1 and F2 litter (i.e. one report for the F1a litter, another report for the F1b litter, etc.); and
- Acquisition of BPS and VO by all pups in the F1c (or PND 50 if either has not occurred).

6. TRACKING ACTIVITY OF PROJECT

The computer system used to keep record of all data related to the project shall be approved by the NTP COTR.

7. PROTOCOL CHANGES

Prior to changes in the protocol, the NIEHS COTR shall be consulted and agree to changes in writing. The change and the reason for the change shall be put in writing in the form of an amendment, signed by both the contract lab Study Director and the NTP COTR, and maintained with the original approved protocol.

NOTE- Determination of Affected Sex:

Determination of Affected Sex – Crossover Mating and Dominant Lethal Testing

If a decrease in fertility and/ or fecundity has been attributed to test article exposure (e.g. a decrease of 2 or more pups per litter), or as otherwise requested by the COTR, the Contractor shall conduct a cross-over mating. A cross-over mating shall consist of (1) mating all the high dose males with test article naive nulliparous females; and (2) mating all high dose females with control males. Animals shall be fed NIH-07 diet (or other if specified by the protocol). Exposure to test articles by food, water or gavage shall be suspended for the duration of mating. (If a dietary study, animals shall be given control diet during this mating period.) Test article naïve nulliparous females shall be purchased in a number sufficient for there to be one-to-one mating pairs. Test article naïve nulliparous females shall be mated with the high dose males when they are sexually mature at 11 weeks of age. Mating pairs of one male and one female, shall be cohabitated until evidence of mating (vaginal plug or vaginal sperm) is found or 7 days, whichever comes first. Litters from the cross-over mating shall be evaluated through PND 4. The following data shall be collected: number of females plugged/sperm positive, number of females delivering a litter, number and sex of live and dead pups on PND 0, 1 and 4. (AGD may be collected if the protocol specifies.) Dams and pups shall be terminated on PND 4 and corpora lutea counted. The high dose and control males shall be terminated if it is determined by the NTP COTR that no further testing of them (such as dominant lethal test) is required. In some cases, additional controls may be required for cross-over mating evaluations.

If a substantial male contribution to the adverse reproductive effect is indicated by the cross-over mating, a male dominant lethal test shall be conducted at the direction of the NTP COTR. If required, the dominant lethal test shall be conducted as soon as possible following completion of the crossover mating so males shall be continually dosed in the interim. High-dose and vehicle control males shall be mated to test article naïve nulliparous females. A sufficient number of naive nulliparous females must be purchased to provide two females for each male (paired 1 male:2 females at the same time). Females shall be 11 weeks old at the time of mating. Females shall be evaluated for the presence of vaginal sperm or a sperm plug daily for the 5 day mating period. Females shall be separated from the males after evidence of mating is found or after 5 days, whichever comes first. Dosing shall be suspended during the mating period. Females shall be euthanized in late gestation (GD19) for analysis of uterine contents for total implantation sites, live fetuses, resorption sites, and dead embryos and fetuses. Corpora lutea shall also be counted. As a general rule, there shall be a sufficient number of pregnant females to provide at least 300 implantation sites per group for evaluation.

Additional Endpoints may be added to the study on a test article specific basis including Toxicokinetics and biological sampling (e.g. maternal/ fetal / pup – tissue levels)

B. PROTOCOL OUTLINE FOR PRENATAL DEVELOPMENTAL TOXICITY STUDY

PROTOCOL OUTLINE FOR THE STUDY OF A TEST ARTICLE IN HARLAN SPRAGUE DAWLEY RATS ADMINISTERED DAILY BY GAVAGE USING THE NTP PRENATAL DEVELOPMENTAL TOXICITY DESIGN [NTP Study Number RXXXX]

1. INTRODUCTION:

1.1. Background and Rationale (test article specific information)

2. **OBJECTIVE:** To characterize the prenatal developmental toxicity of the **TEST ARTICLE** in Harlan Sprague Dawley rats using the NTP design. (Note: Other species may be used as specified by the NTP.)

The prenatal developmental toxicity assessment evaluates test article induced maternal, embryo, and fetal toxicity after exposure to pregnant rats. Typical exposure is from the day of implantation, gestational day (GD) 6, to one day prior to expected parturition, GD 20. Toxicity to the offspring may be in the form of a growth delay, soft tissue or skeletal malformations, or death. A dose range finding study may be needed to determine overt toxicity to the dam and offspring. Generally a dose that elicits minimal maternal toxicity (e.g. reduced body weight, body weight gain) is selected as a maximum tolerable dose. (Note: if NZW rabbits are used, the exposure period is from GD 8-29.)

3. TEST ARTICLE

3.1. Structure, Source:

3.2. Molecular Formula:

3.3. Molecular Weight:

3.4. CAS #:

3.5. Characterization: The composition/purity of the bulk material shall be assayed within 30 days prior to the beginning of the study. A representative sample from each dose for the initial formulation shall be analyzed to confirm concentration and homogeneity. Other analyses shall be conducted as described in the NTP Specifications for Reproductive Studies.

3.6. Route of Administration: Typically by gavage (include dose volume e.g. 2 or 5 ml/kg) but in some cases may be via feed or drinking water**.**

3.7. Vehicle information (w/ CAS #, if appropriate)

4. TEST ANIMALS

4.1. Species, Strain/Stock and Age: Female Harlan Sprague Dawley rats (Harlan Laboratories) shall be between the ages of 11 and 12 weeks of age and mated at the supplier. Body weights shall be approximately 200 to 225 g when mated.

4.2. Diet and Water: Tap water and Feed (NIH-07 or other diet, e.g. low phytoestrogen 5K96, certified, irradiated, analyzed for nutrients/contaminants, and from an NTP-approved vendor) to be provided ad libitum. (NZW Rabbit diet shall be standard pellet diet such as NIH-09, Purina 5322 or other diet specified by the COTR).

4.3. Pre-study evaluation: Animals shall be obtained on or before GD 2. GD 0 is defined as

the day of evidence of mating. Animals shall be examined by a veterinarian prior to study start and release to study.

4.4. **Randomization and Identification:** Prior to dosing, pregnant female rats shall be randomized into treatment groups using a method to minimize the variance in mean body weight, and uniquely identified.

4.5. **Euthanasia:** Discomfort or injury to animals shall be limited, in that if any animal becomes severely debilitated or moribund, it shall be humanely euthanized. Females shall be euthanized on GD 21 using CO_2. Fetuses shall be euthanized by oral administration of pentobarbital (or similar product). This is the best technique for the purpose of this study, which involves critical examination of the internal organ systems of the fetuses. Other euthanasia methodologies/routes of exposure results in artifacts and/or inadvertent damage to internal organs, which interferes with evaluation of fetal morphology.

5. PROCEDURES

5.1. **Study Design: Dose Range-Finding Study (DRF)** *(if required to set dose levels for the prenatal developmental toxicity study; this study may also be used for collection of biological samples for determining test article concentrations in the dam and fetus.)*

5.1.1. Caging

Female rats shall be singly housed throughout the DRF.

5.1.2. Dose levels and number of rats

Rats shall be randomized as described in **Section 4.4** into 4 groups as indicated below. (Provide information on dose selection rationale, if available). (Some DRFs may have 5 dose groups. The number of dams may be increased for some dose groups by 3 or more for the collection of biological samples on GD 18 or 21.)

Group	Dose levels *(mg/kg, ppm, etc)*	No. of Dams
Control (1)		8
2		8
3		8
4		8
	Total Females	**32**

5.1.3. Duration of dosing

Female rats shall be dosed daily from GD 6 to GD 20 (15 days). For gavage studies, dose volume shall be based on the most recent body weight.

5.1.4. In-life endpoints

- Formal (out of cage) clinical observations once daily (for gavage studies this shall occur 1-3 hrs post dose); cage side observations, twice daily (before 10AM and after 2PM), including observation for signs of delivery, moribundity and/or death.
- Female body weights measured daily, starting on GD 5.
- Female feed consumption GD 6-9, 9-12, 12-15, 15-18, 18-21, 6-21. *(include water consumption- if TA is administered in water)*

5.1.5. Necropsy

- Gravid uterine weight, litter size, and number of live and dead fetuses at GD 21 shall be measured. Each fetus shall be weighed, sex determined, and undergo an external examination plus examination for cleft palate. Each fetus will not be uniquely identified, but for each dam, the position of each fetus in the uterine horns, starting at the left end, noting the cervix and moving to the right end, shall be noted.

Rats that do not appear to be pregnant shall be examined for the presence of implants (using ammonium sulfide or potassium ferricyanide) and corpora lutea enumerated. (Rabbit DRF shall include an internal exam for sex determination of the fetus.)

5.2. Study Design: Prenatal Developmental Toxicity Assessment

5.2.1. Caging

Female rats shall be singly housed throughout the study.

5.2.2. Dose levels and number of animals

Female rats shall be randomized as described in **Section 4.4** into 4 groups:

Group	Dose levels	No. of Dams
Vehicle Control		25
Low Dose		25
Mid-Dose		25
High-Dose		25
	Total Females	**100**

A minimum of 25 sperm positive females shall be assigned to each dose group. It is anticipated that a minimum of 20 pregnant rats per group shall be evaluated in the completed study.

5.2.3. Duration of dosing

Dams shall be dosed daily from GD 6 to GD 20, including GD20. For gavage studies, dose volume shall be based on the most recent body weight.

5.2.4. In-life endpoints

5.2.4.1. Dams

- Formal (out of cage) clinical observations once daily (generally 1-3 hours post gavage); cage side observations, including, signs of delivery, moribundity and/or death, twice daily (before 10AM and after 2PM).
- Female body weights measured daily.
- Maternal feed consumption GD 6-9, 9-12, 12-15, 15-18, 18-21, 6-21. *(include water consumption- if Test Article is administered in water).*

5.2.5. Dam Necropsy

5.2.5.1. The following endpoints shall be measured:

- Maternal body, liver, and gravid uterine weights.
- Pregnancy status and the number of corpora lutea. In the gravid uterus, the number of resorptions (early vs late) and live and dead fetuses shall be recorded. Rats that do not appear to be pregnant shall be examined for the presence of implants (using ammonium sulfide or potassium ferricyanide).
- All live fetuses shall be counted, weighed, sexed (visual anogential distance), and examined for external morphological abnormalities, including cleft palate. For each dam, the position of each fetus in the uterine horns, starting at the left end, noting the cervix and moving to the right end, shall be noted. The placenta from live fetuses shall undergo gross observation. Fetuses shall be individually identified so visceral and skeletal findings can be related.
- All of the fetuses shall have sex determined internally and be examined for visceral morphological abnormalities via Staple's technique. Approximately

one-half of the fetuses shall be decapitated prior to dissection. Fetal heads shall be fixed and decalcified in Bouin's solution, and subsequently examined for soft tissue craniofacial alterations.

- All fetuses shall be eviscerated, and the skeletons macerated and stained with Alcian Blue/Alizarin Red S stain. All fetal skeletons shall be examined for skeletal morphological abnormalities.

5.2.6. Data Presentation

Mean values and index of variability (i.e. SE, or SD) for all protocol specified parameters shall be provided to the COTR, including (but not limited to):

- Maternal mean body weights, body weight gains, gravid uterine weight, liver weight, body weight corrected for gravid uterine weight, food consumption and water consumption, if collected (see **Sections 5.1.5** and **5.2.5**)
- Mean litter size, mean body weight of live fetuses by sex and in combination, and total number live/dead pups
- Mean by litter number of resorptions, implants, calculated post implantation loss.
- Both litter and individual fetal incidence by dose group shall be provided for malformation and variation incidence data;
- Malformation is defined as a "permanent structural change that may adversely affect survival, development, or function", while a variation is "a divergence beyond the usual range of structural constitution that may not adversely affect survival or health"
- Data shall be separated by external, visceral, and skeletal changes and by body regions
- Historical control data shall be provided to enhance interpretation of study results
- Data shall be analyzed using standardized parametric and non-parametric tests available within the laboratory's computerized data management system; in addition, malformations may be grouped upon request by the NTP COTR for a syndrome analysis.
- The preferred terminology used to describe the fetal observations shall be consistent with Makris et al. Terminology of Developmental Abnormalities in Common Laboratory Mammals (Version 2) 2009 published in Birth Defects Research Part B, 86:4, pg. 227-327.

Note- Summaries of data shall be provided to the COTR within five days after the following:

- Final necropsy of the DRF study;
- In the main study, final necropsy and completion of the skeletal examinations.

6. TRACKING ACTIVITY OF PROJECT

The computer system used to keep record of all data related to the project shall be approved by the COTR.

7. PROTOCOL CHANGES

Prior to changes in the protocol, the NIEHS COTR shall be consulted and agree to changes in writing. The change and the reason for the change shall be put in writing in the form of an amendment, signed by both the contract laboratory Study Director and the NTP COTR, and maintained with the original approved protocol.

C. PROTOCOL OUTLINE FOR THE NTP MODIFIED ONE GENERATION STUDY

PROTOCOL OUTLINE FOR A STUDY OF (TEST ARTICLE) VIA (EXPOSURE ROUTE) IN HARLAN SPRAGUE DAWLEY RATS USING THE NTP MODIFIED ONE GENERATION DESIGN [NTP Study Number RXXXX]

1. INTRODUCTION:

1.1. Background and Rationale (test article specific information)

2. **OBJECTIVE:** To characterize the reproductive toxicity of the TEST ARTICLE in Harlan Sprague Dawley rats using the NTP modified one-generation (MOG) study design.

The MOG is composed of two interrelated parts: (1) if the acceptable range of dose levels required to avoid excessive general and perinatal toxicity is unknown, a dose range-finding (DRF) study is conducted to determine the maximal dose level that is tolerated by the dam (dosing gestation day 6 through weaning) and that has no impact on perinatal survival until weaning (2) in the definitive MOG, sperm positive females are administered the test material from gestation day 6 through weaning. The subsequent F_1 offspring are then continuously administered the test article via the same route of exposure as the dams. All F_1 animals after PND 4 standardization are taken to adulthood for gross and histopathology examination and can be allocated to various cohorts (e.g. general toxicity, fecundity/fertility, teratology, neuro- or immuno- toxicity endpoints). Histopathology examination of multiple animals per litter increases the power to detect adverse effects (Blystone et al., 2010).

3. TEST ARTICLE

3.1. Structure, Molecular Weight, CAS #, Source, Alternative names, etc

3.2. Characterization: The composition/purity of the bulk material shall be assayed within 30 days prior to study start and at 24 $\pm$ 2 week intervals. A representative sample from each dose of the initial formulated material preparation shall be analyzed to confirm concentration and homogeneity, and subsequent formulation analyses shall be as outlined in the NTP Specifications for Reproductive and Developmental Studies.

3.3. Route of Administration: oral feed or gavage or drinking water (gavage dosing volume, e.g. 2, or 5 ml/kg)

3.4. Vehicle information (w/ CAS #, if appropriate)

3.5. Positive Control: if used (w/ CAS #, Molecular formula, Molecular Weight)

4. TEST ANIMALS

4.1. Species, Strain/Stock and Age: Time-mated (assumed pregnant) female Harlan Sprague Dawley Rats (Harlan Laboratories) (or other specified by the NTP). F_0 female rats shall be sexually mature (11-12 weeks; 200-225 g) and mated at the supplier. Male and female rats shall be sexually mature when mated during the study.

4.2. Diet and Water: Tap water and Feed (NIH-07; certified and irradiated; analyzed for nutrients and contaminants; from an NTP-approved vendor, or other diet e.g. low phytoestrogen diet) to be provided ad libitum.

4.3. Pre-study evaluation: Animals shall be obtained on or before gestation day (GD) 2. Day of positive evidence of mating = GD 0. Animals shall be examined by the veterinarian before being released for study. A sentinel program shall be followed to ensure the health of the animals on study. A sufficient number of animals shall be purchased for this purpose.

4.4. Randomization and Identification: On GD 3, F_0 female rats shall be randomized into treatment groups to minimize the variance in mean body weight, and uniquely identified. F_1 pups (in the Definitive Study) shall be identified by paw tattoo according to number within litter at PND 1 and, when retained beyond PND 4, uniquely identified at weaning.

4.5. Euthanasia: Discomfort or injury to animals shall be limited, in that if any animal, including pre-weaning F_l (or F_2 offspring), becomes severely debilitated or moribund, it (as well as all rats at scheduled necropsy/termination) shall be humanely euthanized.

5. PROCEDURES

5.1. STUDY DESIGN: DOSE RANGE-FINDING STUDY (DRF) *(if required to set dose levels for the definitive MOG study); some studies may also include additional rats for Biological Sampling / tissue level analyses.*

5.1.1. Caging

Dams shall be housed one per cage by dose group. Pups shall remain with their respective dam until euthanasia at weaning (on PND 21 or 28). If weaning is on PND 28, such as for the HSD rat, intervals for measurements shall extend to PND 28.

5.1.2. Dose levels and number of rats

Rats shall be randomized as described in **Section 4.4** into 6 groups and continuously exposed via (feed or gavage) containing the dose indicated below:
(*Typically there will be 8 animals per group. Number of F_0 females may be increased to accommodate biological sampling.)*

Group	**Dose levels** *(mg/kg, ppm, etc)*	**No. of F_0 females**
Control (1)		8 (14)
2		8 (14)
3		8
4		8
5		8
6		8 (14)
	Total	**48 (66)**

5.1.3. Duration of dosing *(and frequency if gavage)*

Females shall be dosed daily/continuously from GD 6 through weaning (i.e. up to ~6 weeks *or longer depending on the SOW*). PND 0 is defined as the day that all pups within a litter have been delivered. For gavage studies, dose volume shall be based on the most recent dam and pup body weight. Note- for direct dosing to pups in a gavage study, pup dosing begins on PND 12.

5.1.4. In-life endpoints

- Formal (out of cage) clinical observations once daily; cage side observations, twice daily (before 10AM and after 2PM) including signs of delivery, moribundity and/or death.
- Dam body weights recorded daily during gestation (day of randomization onwards); PND 1, 4, 7, 14 and 21. All weights are to be reported as well as gestational/post-natal weights for the following intervals: GD 3-6, 6-9, 9-12, 12-15, 15-18, 18-21, and 6-21; PND 1-4, 4-7, 7-14, 14-21,1-21. (Extend to 28 if necessary.)
- Dam feed consumption measured for *(include water consumption- if Test Article is administered in water)* GD 3-6, 6-9, 9-12, 12-15, 15-18, 18-21, 6-21, and PND 1-4, 4-7, 7-14, 14-21, 1-21. (Extend to 28 if necessary.)
- Number of live and dead pups delivered by sex and by litter and total on PND 0.

- Number and weight of live pups by sex & by litter & total on PND 1, 4, 7, 14, 21, 28.
- Number of pregnant and non-pregnant rats; duration of pregnancy

5.1.5. Biological sample collection (if applicable) (Dose groups & N may vary)

On GD 18, confirmed pregnant dams (3/group at 0, low and high doses) shall be euthanized and the following collected: maternal plasma isolated from trunk blood, as much as possible, target 3 ml; amniotic fluid (pooled by litter, target 1.0 ml; fetuses (pooled by litter, frozen in liquid nitrogen). Store at -20 C. On PND 4, 3 dams and their pups (3 dams/group at 0, low and high doses) shall be euthanized and the following collected: maternal plasma, as much as possible, target 3 ml; whole pup in a manner appropriate for chemical analysis (3 per sex/litter). Store at -20 C. Tissues and plasma shall be sent to a designated NTP Chemistry contractor for test article analysis.

5.1.6. Euthanasia

Females and pups shall be euthanized at weaning (or sufficiently after expected littering date, if no evidence of pregnancy). Rats that produced litters shall be discarded without examination. Rats that do not appear to be pregnant and do not deliver shall be examined for the presence of uterine implants (using ammonium sulfide or potassium ferricyanide) and ovarian corpora lutea enumerated.

5.2. STUDY DESIGN: DEFINITIVE STUDY

5.2.1. Caging

Dams shall be housed one per cage by dose group. Pups shall remain with their respective dam until weaning on PND 21 or 28 (or euthanasia). After weaning, juveniles shall be group housed by litter and sex (up to 4 per cage as appropriate for their size). If selected for the F_1 fertility or prenatal toxicity cohort, rats shall be housed as pairs of one male and one female per cage from the same dose group (up to 15 days, or until positive evidence of mating). After evidence of mating, or after 15 days if no evidence of mating, the pairs shall be separated and individually housed until necropsy.

5.2.2. Dose levels and number of animals

Rats shall be randomized as described in **Section 4.4** into 4 groups:

Group	Dose levels[a] (mg/kg, ppm, etc)	No. of F_0 females
Vehicle Control		25
Low Dose		25
Mid-Dose		25
High-Dose		25
	Total F0	**100**
a: F_1 offspring shall also be administered the test article post-weaning at the same dose as their parents		

5.2.3. Duration of dosing

Depending on the dosing route, F_0 rats shall be administered control- or compound-containing feed ad libitum continuously (or water) containing the test article or dosed daily by gavage- include dose volume [e.g. 2 or 5 mL/kg]), 7 days a week, from GD 6 throughout gestation and until necropsy.

F_1 generation pups shall be exposed via nursing/eating dosed feed. After weaning the offspring shall be administered control- or compound-containing feed (at the same dose level the dam received) ad libitum continuously, 7 days a week until euthanasia. Rats allocated to a prenatal toxicity or fertility cohort shall be administered the test compound in the diet during breeding and gestation until scheduled necropsy. *For gavage studies, dose volume shall be based on the most recent dam and pup body weight. Note- for*

direct dosing to pups, pup dosing begins on PND 12 in a gavage study.

Phase of study	Total days on feed
F_0 females-until weaning	~36 days
F_1 Sub-chronic cohort	~91 days
F_1 Teratology cohort	~ 106 days
F_1 Breeding cohort	~ 136 days; ~ 164 w/crossover
F_1 Neurotox cohort	TBD ~91 days
F_1 Immunology cohort	~50 days

5.2.4. F_1 production, randomization and disposition

F_1 pups shall be uniquely identified on PND 1 by paw tattoo. When possible, the F_1 litters shall be standardized to 8 pups (4 male and 4 females /litter when possible) on PND 4 (or 10 pups (5+5) if all cohorts are conducted). The day pups shall be randomly allocated (by treatment) to the following cohorts depends on cohorts conducted:

Potential Cohorts*	Number of male and female pups/litter	Total number of male pups	Total number of female pups
Toxicity	1 male and 1 female	10	10
Prenatal Toxicology	1 male and 1 female	20	20
Fertility	1 male and 1 female	20	20
Developmental Neurotoxicology	1 male and 1 female	20	20
Immuno toxicity	1 male and 1 female	20	20
* Extra offspring from each of these cohorts may be used for "special studies" (i.e. clinical pathology, mammary gland evaluation, biological sampling, TK/ADME, etc). Note- if additional litters are available or if all cohorts are not conducted, then the number of pups allocated to other groups may be increased.			

At PND 4 some studies may include collection of pup mammary glands. The inguinal mammary glands from up to ten males/dose level and ten females/dose level randomly selected from the F_1 culls on PND 4 (from different litters) shall be collected on PND 4 and whole mounted for subsequent assessment of abnormal mammary gland development.

Non-selected F_1 pups shall be euthanized on PND 4. All pups and rats at necropsy shall be euthanized by Contractor-IACUC-approved procedures acceptable to the NTP COTR. *Note- if it is not possible to determine sex on PND 4 (i.e. apparent skewed sex ratio), culling shall be delayed until weaning.*

5.2.5. In-life endpoints (applicable to all cohorts or as appropriate; if weaning occurs at PND 28 instead of PND 21, additional intervals for certain measurements must be included to accommodate the time between PND 21-28.)

5.2.5.1. F_0 dams

- Formal (out of cage) clinical observations once daily; cage side observations, including, moribundity and/or death, twice daily (before 10AM and after 2PM)
- Female body weights daily during gestation and PND 1, 4, 7, 10, 13, 16, 19 and 21; at termination. Body weight changes to be reported for the following intervals: GD 3-6, 6-9, 9-12, 12-15, 15-18, 18-21, 0-21, PND 1-4, 4-7, 7-10,

10-13, 13-16, 16-19, 19-21; PND 1-16

- Feed consumption (and water consumption-if administered in the water) twice weekly (when not mated/pregnant); twice weekly is defined as every three, then 4 days; GD 3-6, 6-9, 9-12, 12-15, 15-18, 18-21; PND 1-4, 4-7, 7-10, 10-13, 13-16, 16-19, 19-21 and PND 1-21
- Number of pregnant females and duration of pregnancy.

5.2.5.2. F_1 offspring

- PND 0: Number of live and dead offspring (by litter, sex, and total)
- PND 1: Number of live and dead offspring (by litter, sex, and total); anogenital distance (AGD) and corresponding pup weight.
- PND 4: Number of live and dead pups, sex, pre-cull weight (by litter, sex, and total).
- Presence and type of developmental anomalies in the pups (e.g. clinical observations, gross external malformations).
- Number and weight of pups on PND 4 (post cull), 7, 10, 13, 16, 19, 21 *(and PND 28 for HSD rats)* (by pup, sex and litter). Pup body weight gain changes by sex to be reported for the following intervals 1-4 pre cull, 4 (post cull)-7, 7-10, 10-13, 13-16, 16-19, 19-21, post cull 4-21
- Males: Presence of areolae on PND 13, testicular descent (PND 14-30), balano-preputial separation ((BPS) PND 35 until acquisition; the NTP-COTR shall be consulted if BPS has not occurred by PND 50) and body weight on day of acquisition.
- Females: day of vaginal opening (PND 23 until acquisition; the COTR shall be consulted if VO has not occurred by PND 42) and body weight on day of acquisition and estrous cyclicity (PND 77 +/- 3 days and continuing for 16 days).
- After weaning: daily out of cage clinical observations; cage-side observations, twice daily (before 10AM and after 2PM); weekly body weights and cage feed consumption *(and water consumption if the Test Article is supplied in the water)*

5.2.6. F_0 Necropsy

- F_0 parental rats shall be necropsied after F_1 weaning has been completed. Animals shall be subjected to external examination and uterine implantation scars enumerated. Gross lesions and representative control tissues shall be retained, processed to slides and examined histopathologically. Rats that do not appear to be pregnant and do not deliver shall be examined for the presence of implants (using potassium ferricyanide) and corpora lutea enumerated.

5.3. TOXICITY COHORT

5.3.1. Allocation

- F1 offspring shall be randomly allocated to the Toxicology cohort as described in Section 5.2.4.

5.3.2. In-life Endpoints

- See Section 5.2.5

5.3.3. Toxicological Parameters to be Evaluated: [Endpoints evaluated may vary depending on the test article under study]

- **F_1 Clinical Laboratory Studies**

Rats shall be bled for the terminal collection for clinical laboratory studies (PND 112 $\pm$ 4).

All animals of a sex shall be bled on the same day.

Hematology
Erythrocyte count
Hemoglobin concentration
Hematocrit (Packed cell volume)
Mean corpuscular volume
Mean corpuscular hemoglobin
Mean corpuscular hemoglobin concentration
Leukocyte count
Leukocyte differential
Reticulocyte count
Platelet count
Morphologic assessment erythrocytes, leukocytes and platelets

Clinical chemistries
Total Protein
Albumin
Urea Nitrogen (BUN)
Creatinine
Alanine Aminotransferase (ALT)
Sorbitol dehydrogenase (SDH)
Alkaline Phosphatase (ALP)
Total Bile Acids
Glucose
Creatine Kinase (CK)
Cholesterol
Triglycerides

The results of all automated measurements for clinical pathology (unaudited data) shall be reported to the NTP within seven calendar days after sample collection.

- **Blood For Micronuclei**
  Samples of blood (~200 μl) shall be collected in EDTA from rats at termination. Samples are to be refrigerated immediately after collection and remain refrigerated until shipped the day of collection. The samples shall be shipped refrigerated (not frozen) for overnight delivery to an NTP-designated laboratory for micronuclei determination.

- **Sperm Motility, Count and Vaginal Cytology Evaluations**
  Sperm motility, count and vaginal cytology evaluations (SMCVCE) shall be conducted on core study rats in accordance with the protocol in the Appendix to the Specifications. The NTP Project Officer shall be notified at the end of the first 70 days of the study regarding mortality, body weight changes and clinical signs of toxicity for use in determining the treatment groups to be used in the SMCVCE. *Vaginal cytology shall be assessed on all cohorts (as applicable); sperm analyses will not need to be conducted if it is assessed in the fertility and/or prenatal cohorts.*

- **Mammary Glands**
  In some studies, the inguinal mammary glands from one F_1 offspring/sex/litter at the time of vaginal opening (female) or PND 21 or 28 (male) shall be collected and whole mounted (up to ten/sex/dose level at the appropriate interval). These animals shall

be given a gross examination and tissues with gross lesions (and comparative controls) shall be fixed and retained for subsequent histopathological evaluation. *Note- this can only be conducted if extra animals are available.*

- **Necropsy and Histopathologic Evaluation (PND 112 ± 4)**

  Organ weights shall be determined from all animals surviving until the end of the study. Those organs to be weighed are: Liver, thymus, right kidney, testes, epididymides, seminal vesicles w/coagulating glands, heart, prostate, and lungs. Organs shall be weighed to the nearest 10.0 mg except for testes, epididymides, prostate, and thymus that shall be weighed to the nearest 1.0 mg.

  A complete necropsy shall be performed on all treated and control animals that either die or are euthanized, and all tissues as listed in NTP Specifications shall be saved. All tissues required for complete histopathology as listed in the NTP Specifications shall then be trimmed, embedded, sectioned and stained with hematoxylin and eosin for possible histopathologic evaluation. This shall be done for all animals in all groups.

  Gross lesions shall be examined in all animals in all treatment and control groups. A complete histopathologic evaluation shall be done on all control animals, all animals in the highest treatment group with at least 60% survivors at the time of sacrifice, plus all animals in higher treatment groups. Treatment-related lesions (target organs) shall be identified and examined in lower treatment groups to a no-effect level. For all natural death/moribund sacrifice animals a complete histopathologic evaluation shall be performed.

LIST OF TISSUES FOR COMPLETE HISTOPATHOLOGIC EVALUATION

Adrenal glands
Brain (see test article specific protocol for details, up to 7 sections inc. frontal cortex, basal ganglia, parietal cortex, thalamus, and medulla)
Clitoral glands
Esophagus
Eyes
Femur, including diaphysis with marrow cavity & epiphysis (femoral condyle with epiphyseal cartilage plate, articular cartilage and articular surface)
Gallbladder (mouse)
Gross lesions
Harderian glands
Heart and aorta
Intestine, large (cecum, colon, rectum)
Intestine, small (duodenum, jejunum, ileum)
Kidneys
Liver (2 sections including left lateral lobe and median lobe)
Lungs and mainstem bronchi
Mammary gland with adjacent skin
Muscle, thigh (if neuromuscular signs were present)
Nasal cavity and nasal turbinates (3 sections)
Ovaries
Pancreas
Parathyroid glands
Pituitary gland
Preputial glands
Prostate
Salivary glands
Seminal vesicles
Coagulating glands
Sciatic, tibial (rat) and trigeminal nerves (if neurological signs were seen)
Spinal cord
Spleen
Stomach (forestomach and glandular)
Testes with epididymides
Thymus
Thyroid Gland
Tissue masses
Trachea
Urinary bladder

Lymph nodes - mandibular and mesenteric Uterus

In some studies, liver sections for potential molecular analyses may be collected and shipped to an NTP-designated laboratory.

- **Urinalysis** (OPTIONAL NOT PART OF STANDARD DESIGN)
  Urine analyses shall be performed in F_1 male and female rats during week 12 (PND 112) (a 16-hour urine collection following at least 2 consecutive treatment days).

  Appearance
  Volume
  Specific gravity
  Microscopic assessment of sediment
  Protein
  Glucose
  Creatinine

  [***Include only those assays below that are appropriate for the test article under study]***
  N-acetyl-B-glucosaminidase (NAG)
  Lactate dehydrogenase (LDH)
  Alkaline phosphatase (AP)
  Aspartate aminotransferase (AST)

5.4. PRENATAL TOXICOLOGY COHORT

5.4.1. Allocation

- F_1 offspring shall be randomly allocated to the prenatal toxicity cohort as described in Section 5.2.4. Vaginal cytology shall be initiated on week 11 (prior to cohabitation). After 16 days (week 13), one male and one female from the same dose group, and avoiding sibling mating, shall be cohabitated. After evidence of mating (or after 15 days if no evidence of mating), male and females shall be separated and females shall be terminated on GD 21. (For gavage studies, last day of dosing is GD20.)

5.4.2. In-life Endpoints

- See Section 5.2.5 for endpoints during gestation
- Time to mating (pre-coital interval)

5.4.3. F_1 Dam Necropsy

The following endpoints shall be measured:

- Maternal body, liver, adrenal, ovarian, and gravid uterine weights.
- Pregnancy status and the number of corpora lutea. In the gravid uterus, the numbers of deaths (resorptions (early vs. late) and/or dead fetuses) and live fetuses shall be recorded. Rats that do not appear to be pregnant shall be examined for the presence or evidence of implants (using potassium ferricyanide or other appropriate method).
- All live fetuses shall be counted, weighed, sex determined (visual anogenital distance), and examined for external morphological abnormalities, including cleft palate. For each dam, the position of each fetus in the uterine horns, starting at the left end, noting the cervix and moving to the right end, shall be noted. The placentas from live fetuses shall undergo gross observation.
- All of the fetuses shall be internally sexed and examined for visceral morphological abnormalities via Staple's technique. Approximately one-half of the same fetuses shall be decapitated prior to dissection. Fetal heads shall be fixed and decalcified in Bouin's solution, and subsequentaly examined for soft tissue craniofacial alterations.

- All fetuses shall be eviscerated, and the skeletons macerated and stained with Alcian Blue/Alizerin Red S stain. All fetal skeletons shall be examined for skeletal and morphological abnormalities.

5.4.4. F_1 Male Necropsy

- Males shall be necropsied after the cohabitation period and shall be subjected to a gross examination.

- Organ weights:

Adrenal glands (paired)	Dorso-lateral Prostate
Epididymis, right and left	Ventral Prostate
Cauda Epididymis, right and left	Thyroid gland
Testis, right and left	Preputial glands[a]
Seminal Vesicles (w/ Coagulating Glands)	
Levator ani bulbocavernosus[a]	
a: Only collected and weighed when an antiandrogenic effect is expected (e.g. observed increased incidence of areolae, delay in BPS)	

5.5. FERTILITY COHORT

5.5.1. Allocation

- F_1 offspring shall be randomly allocated to the Fertility cohort as described in Section 5.2.4. Vaginal cytology shall be initiated on week 11 (prior to cohabitation). After 16 days of estrus cyclicity measurements (week 13), one male and one female from the same dose group, avoiding sibling mating, shall be cohabitated. After evidence of mating (or after 15 days if no evidence of mating), male and females shall be separated and females shall be allowed to litter. Pups shall remain with their dams until weaning.

5.5.2. In-life endpoints

- See Section 5.2.5

F_1 dams

- Time to mating (pre-coital interval)
- Formal (out of cage) observations once daily; cage side observations, including, moribundity and/or death, twice daily (before 10AM and after 2PM) *(NOTE- twice weekly is defined as every three, then 4 days)*
- Female body weights daily during gestation and PND 1, 4, 7, 10, 13, 16, 19 and 21; at termination. Body weight changes to be reported for the following intervals: GD 0-3, 3-6, 6-9, 9-12, 12-15, 15-18, 18-21, 0-21, PND 1-4, 4-7, 7-10, 10-13, 13-16, 16-19, 19-21; PND 1-16
- Feed consumption (and water consumption if Test Article is administered in the water) twice weekly (when not mated/pregnant); GD 0-3, 3-6, 6-9, 9-12, 12-15, 15-18, 18-20; PND 1-4, 4-7 7-10, 10-13, 13-16, 16-19, 19-21 and PND 1-16
- Number of pregnant females and duration of pregnancy.

F_2 offspring

- PND 0: Number of live and dead offspring (by litter, sex, and total)
- PND 1: Number of live and dead offspring (by litter, sex, and total); anogenital

distance (AGD) and corresponding pup weight.

- PND 4: Number of live and dead pups, sex, pre-cull weight (by litter, sex, and total).
- Presence and type of developmental anomalies in the pups (e.g. clinical observations, gross external malformations). Generally cull to a maximum of 8 pups (4 males and 4 females where possible).
- Number and weight of pups on PND 4 (post cull), 7, 10, 13, 16, 19, 21 *(and PND 28for HSD rats)* (by pup, sex and litter). Pup body weight gain changes by sex to be reported for the following intervals 1-4 pre cull, 4 (post cull)-7, 7-10, 10-13, 13-16, 16-19, 19-21, post cull 4-21
- Males: Presence of areolae on PND 13, Optional Endpoints: testicular descent (PND 21-30), balano-preputial separation (BPS; PND 38 until acquisition; the NTP-COTR shall be consulted if BPS has not occurred by PND 50) and body weight on day of acquisition.
- Females: Optional Endpoints: day of vaginal opening (PND 23 until acquisition; the COTR shall be consulted if VO has not occurred by PND 42) and body weight on day of acquisition and estrous cyclicity (PND 76 to PND 91). *(NOTE-these endpoints shall be conducted only if the F2 pups are retained past weaning.)*

5.5.3. F_1 Parental Necropsy

- Females shall be necropsied after the F_2 offspring have been euthanized

- Males shall be necropsied after potential effects on fertility have been ascertained *(see Section 5.5.5)*. If areolae are noted on male rats at PND 13, the ventral surface of male rats shall be shaved and nipples counted.

- Trunk blood shall be collected and sera isolated (target volume 3mL) and frozen for potential hormone (or other potential) analyses as directed by the COTR.

The following endpoints shall also be measured:

- Leftt cauda epididymal spermatzoa measurements: number, density, motility.

- Left testicular measurements: spermatid head count. *Note- in the case of unilateral effects on organ weights, or presence of gross lesions, pathology takes precedence over sperm assessments in selection of right or left testis/ epididymis.*

- Organ weights:

Adrenal glands (paired)	Dorso-lateral Prostate
Epididymis, right and left	Ventral Prostate
Cauda Epididymis, right and left	Ovaries, right and left
Testis, right and left	Thyroid Gland
Seminal Vesicles (w/ Coagulating Glands)	Preputial gland[a]
Levator ani bulbocavernosus[a]	
a: Only be collected and weighed when an antiandrogenic effect is expected (e.g. decrease in AGD, observed increased incidence of areolae, or a delay in BPS)	

- Fix and process the following tissues[a] per NTP specifications:

Adrenal Glands	Pituitary

Vagina, cervix, and uterus (together)	Thyroid Gland
Epididymis, right [b]	Gross lesions
Testis, right [b]	Mammary gland/ nipples: inguinal 4&5
Seminal Vesicles and Coagulating Glands (together)	
Dorsal Prostate	
Ventral Prostate	
Ovaries[c]	

a: All tissues shall be fixed in formal with the exception of the testes and epididymides which shall be fixed in modified Davidsons
b: If the rat exhibits a gross findings on the left, rather than the right, collect, process, and examine the left tissues for histopathology See **Section 5.2.8.2**
c: Step sectioned and primordial follicles enumerated

Fixed tissues shall be processed to slides and stained with hemotoxylin and eosin. The high and control groups shall be examined for histopathological changes. If high dose effects are found, all the remaining dose groups shall be examined for these and any related changes as defined by the COTR.

5.5.4. F_2 Necropsy

Pups shall be necropsied on PND 21 or 28 and subjected to an external and internal examination. Gross lesions and comparative controls shall be retained and processed for histopathological examination.

5.5.5. Determination of Affected Sex – Crossover Mating

If a decrease in fertility and/ or fecundity has been attributed to test article exposure (e.g. a decrease of 2 or more pups per litter), or as otherwise requested by the COTR, the Contractor shall conduct a cross-over mating. A cross-over mating shall consist of (1) mating all the high dose males with test article naive nulliparous females (i.e. newly purchased); and (2) mating all high dose females with control males. Exposure to test articles by food, water or gavage shall be suspended for the duration of mating. Test article naïve nulliparous females shall be purchased in a number sufficient for there to be one-to-one mating pairs. Test article naïve nulliparous females shall be mated with the high dose males when they are sexually mature at 11 weeks of age. Mating pairs of one male and one female, shall be cohabitated until evidence of mating (vaginal plug or vaginal sperm) is found or 7 days, whichever comes first. Litters from the cross-over mating shall be evaluated through PND 4. The following data shall be collected: number of females plugged/sperm positive, number of females delivering a litter, number and sex of live and dead pups on PND 0, 1 and 4. Dams and pups shall be terminated on PND 4 and corpora lutea counted. The high dose and control males shall be terminated if it is determined by the NTP COTR that no further testing of them (such as dominant lethal test) is required. In some cases, additional controls shall be required.

5.6. NEUROBEHAVIORAL COHORT

Animals shall be evaluated in a battery of tests to provide a functional evaluation of the development of the central nervous system. Animals shall be evaluated at different ages typically using tests to evaluate motor activity, auditory startle, and learning & memory. Neuropathology shall also be conducted. Neurotoxicity tests shall vary based on the test material.

5.6.1. Allocation

F1 offspring shall be randomly allocated to the Toxicology cohort as described in Section 5.2.4.

5.6.2. In-life endpoints
See Section 5.2.5

5.6.2.1. Motor activity
Motor activity shall be monitored by an automated activity recording apparatus around weaning and 60 (±2) days.

5.6.2.2. Auditory startle test
An auditory startle habituation test shall be performed on the offspring around the time of weaning and around day PND 60.

5.6.2.3. Learning and memory test
A test of learning and memory shall be conducted around the time of weaning and around PND 60.

5.6.2.4. Neuropathology
Neuropathological evaluation shall be conducted on animals on postnatal day 11 and at the termination of the study. At 11 days of age, one male or female pup shall be removed from each selected litter such that equal numbers of male and female offspring are removed (N = 10/sex/dose) and euthanized for neuropathological analysis. The pups shall be killed by exposure to carbon dioxide and immediately thereafter the brains shall be removed, weighed, and immersion-fixed in an appropriate aldehyde fixative. For some studies, the COTR will indicate if additional animals are to be removed for collection of specified brain area weights. In some studies, the COTR may specify that whole body perfusions are to be performed.

At the termination of the study, one male or one female from each designated litter shall be euthanized by exposure to carbon dioxide and immediately thereafter the brain shall be removed and fixed for neuropathological evaluation. For some studies whole body perfusion may be required for these analyses.

5.7. IMMUNOTOX COHORT

Animals (specify females only or both sexes) shall be allocated as outlined in Section 5.2.4 and transferred on PND 25 to an NTP designated contractor with sufficient amount of dosing material / control and dosed feed to last until PND 50. Study scheduling and animal shipment must be coordinated with the NTP Immunology Discipline Leader or a designee and the NTP designated immunotoxicology contractor prior to mating the F0 females. Contact information (including phone and email) for the individual coordinating the shipment, and serology studies attesting to the health status of sentinel animals housed in the same room as the study animals must be received at the NTP designated Immunotoxicology contract laboratory a minimum of 21 days prior to shipment of the F1 Immunotoxicology cohort. The Immunotoxicology work shall be conducted using a separate stand-alone protocol.

6. Data Presentation (Each SOW will be customized to reflect optional endpoints)

Mean values and index of variability (i.e. SE, or SD) for all protocol specified parameters shall be provided to the COTR, including (but not limited to):

- Mean body weights, body weight gains, clinical signs, food and water consumption (see **Sections 5.1.5** and **5.2.5**)
- Male and female fecundity and fertility indices
- Mean litter size, mean body weight of live pups (by sex, and combined) and total number

live/dead pups.

- Pup survival on PND 1-4, 4-7, 7-14, 14-21
- AGD (by sex, nested by litter and co-varied by body weight)
- Mean number of areolae and nipples (by litter)
- Results of external, visceral and skeletal fetal examinations
- Organ weights

Note- Summaries of data shall be provided to the COTR within five days after each of the following:

- End of the DRF study;
- Delivery of the last individual dam for each F0 and F1 litter (i.e. one report for the F1a litter, another report for the F1b litter if generated, etc.); and
- Acquisition of BPS and VO by all pups evaluated (or PND 50 if either has not occurred).

7. TRACKING ACTIVITY OF PROJECT

The computer system used to keep record of all data related to the project shall be approved by the NTP-COTR.

8. PROTOCOL CHANGES

Prior to changes in the protocol, the NTP COTR shall be consulted and agree to changes in writing. The change and the reason for the change shall be put in writing in the form of an amendment, signed by both the contract lab Study Director and the NTP COTR, and maintained with the original approved protocol.

9. REFERENCES

Blystone, CR, Kissling, GE, Bishop, JB, Chapin, RE, Wolfe, GW, Foster, PM. Determination of the di-(2-ethylhexyl) phthalate NOAEL for reproductive development in the rat: importance of the retention of extra animals to adulthood. Toxicol Sci. 2010 Aug; 116(2):640-6.

XI. DATA COLLECTION AND SUBMISSION

Study data shall be collected using a computerized real-time data collection system. For reproductive and developmental toxicology data, NTP is currently in the process of implementing a customized data collection and management system (Instem's Provantis). Until that time, the Task Order/Work Assignment shall specify whether the data shall be collected using the contract laboratory's pre-approved data system, an NTP provided data system or combination. The NTP's current data system is the Toxicology Data Management System (TDMSE), which has historically been used to collect data from subchronic and chronic toxicity studies. This system does not have a specific module for the collection of developmental and reproductive toxicology data. (Prior to the implementation of NTP's Provantis system, this Specifications document for reproductive studies will be amended to provide further direction on data collection and management.)

A. DATA COLLECTION

If the protocol specifies that the TDMSE/LDAS collection shall be used for certain aspects of a study, refer to the specifications contained in Section X of The Specifications for the Conduct of Studies to Evaluate the Toxic and Carcinogenic Potential of Chemical, Biological and Physical Agents in Laboratory Animals for the National Toxicology Program, 2010.

The NTP is currently in the process of procuring a hosted Provantis data management system. When the system becomes functional, these Specifications shall be updated to provide information about the use of this system.

In some cases, the laboratory's data collection system may be used. If the protocol specifies that the laboratory's data collection system shall be used, that system must be approved by the NTP COTR.

Independent of which data collection system is specified, the data collection system must include: customizable protocol input for unique study design; the ability to track maternal animals and individual pups from specific litters; a hierarchical security structure to control access to individual data files (e.g. technician clearance, and study director clearance); real time dose calculation for gavage studies based on body weights and dosing volume; integration of measuring apparatus to input values directly into the system without manual reentry; ability to create summary reports for all major study endpoints during the course of the study including standard parametric and non-parametric analyses for monitoring study progress. The system shall be able to output final data as Comma Separated Values (CSV), SAS datasets, excel spread sheets or other output that is compatible with NTP systems so that the NTP can perform customized statistical analysis to include in final reports or NTP Technical Reports. The protocol may specify a specific format for the file type and format of the outputted final data. The testing laboratory shall be responsible for qualification/calibration of balances and other equipment interfaces used for input to the data management system.

B. SUBMISSION OF REPORTS AND DATA

1. Submission of Study Reports

Laboratory reports (including final study reports and prestart chemistry reports) shall be submitted electronically on a CD-R CD (or other mutually agreed upon multimedia format, eg., DVD), along with a single paper copy of each report. For some studies, the laboratory may be instructed to submit more than one hard copy of the report; this will be communicated by the COTR. CDs shall be prepared as follows:

- Reports shall be saved into a single searchable PDF. Do not use password protection or set any document property restrictions. The file is then burned to a CD.

- The original CD shall be examined by the study laboratory for completeness, accuracy, and quality prior to submission to the NTP. The PDF file shall contain either a memo with signature or a digital signature from the study director indicating that the PDF is an exact copy of the report. This shall be the first page or on the first page of the PDF file.

- CDs shall be submitted boxed and labeled to prevent mix-up and damage. Each CD must be labeled with appropriate title, CAS#, NTP Study number (such as R#, T# or C#), and laboratory study number and name.

- Should the report require amendment then the report and an updated CD with all revisions are to be submitted to the NTP.

2. Study Data

a) Submission of Data to the NTP Archives

1) **All original** source documentation is the property of the NTP and shall be sent to the NTP Archives. Only that correspondence related to the technical conduct of a study shall be included. Site visit and Annual Program Review reports and responses to action items shall not be submitted to the NTP Archives.

2) Inventory of the records sent shall be recorded on the NTP Archives Inventory/Index lists as shown on following pages. The Inventory/Index lists shall be prepared by the study laboratory and shall accompany the records to the NTP Archives. Each Section in the NTP Archives Inventory/Index and subpart thereof shall be separated by a stiff paper or cardboard divider or shall be contained in a file folder. A tab affixed to the divider or a label on the file folder shall identify the section and subpart. The laboratory shall provide the folders or dividers and boxes for the records they submit.

3) The records pertaining to a study shall be sent after the pathology materials are sent to the archives. If records from a study are shipped in parts at different times, Inventory/Index lists shall accompany each shipment. The Archives clerk will prepare and maintain a master copy of the Inventory/Index for this study to show all receipts of records from the contractor laboratory, computer data forms, or other sources.

4) Pathology materials (slides, blocks, tissues) shall be sent to the NTP Archives as specified in Section VII.I. and in study specific SOWs under Milestones and Deliverables. Pathology materials shall not be placed in the same box with original study documents.

5) A copy of all final reports reviewed by the laboratory's QAU and submitted to the NTP for a study shall be submitted to the NTP Archives. Amendments to reports must also be submitted to the Archives.

6) A copy of each SOP (including revisions) in effect and used during the conduct of each study shall be submitted. (Electronic copies rather than hard copies are acceptable if the laboratory prefers.)

7) Archival materials shall be appropriately boxed and shipped prepaid to the Archives. The following Inventory/Index organization scheme will be used:

NTP Archive Inventory/Index - Archive Records for Each Study

Section I. General

Section II. Test Article Records

Section III. Study Type (to be indicated on each set of forms:
Example - Range-finding Study, Developmental Toxicity, RACB, etc

NTP Archive Inventory/Index

TEST ARTICLE _____ CAS NO. _____

LABORATORY _____ EXP NO. (C #) _____

ADDRESS _____ TDMS NO. _____

CONTRACT NO. _____

SECTION I. - **GENERAL**

BOX # or Date Sent

Other Location or Comment

Archive Location

COLUMN HEADERS AND THEIR DESCRIPTIONS:

Box # or Date Sent
Include the box number for the current shipment in which the listed data/materials are included. If the data/materials listed were submitted in a prior shipment, indicate the date of that prior shipment.

Other Location or Comment
If data/materials listed have been included in another location, the other location is to be identified here. This space is also included for additional explanatory comments.

Archive Location
This column will be completed by NTP Archival staff when storing the listed data/materials.

	BOX # or Date Sent	Other Location or Comment	Archive Location
A. Personnel (indicate time interval & job) 1. List of Key/Critical Personnel who participated in the study 2. Identify Consultants (by name and full address; specify work performed)			
B. List of Subcontractors (Identify each by name and full address; specify work performed)			
NOTE: Records which pertain to multiple areas within one section or to multiple sections and cannot be separated conveniently to fit into the filing scheme are to be filed in one section and referenced in the other section(s) to which they pertain.			

NTP Archive Inventory/Index

TEST ARTICLE _____ CAS NO. _____

LABORATORY _____ EXP NO. (C #) _____

ADDRESS _____ NTP STUDY NO. _____

CONTRACT NO. _____

SECTION II - TEST SUBSTANCE RECORDS

STUDY TYPE(S) _____	BOX # or Date Sent	Other Location or Comment	Archive Location
A. Identity (Manufacturer, Lot(s), Date)			
B. Characterization			
C. Bulk Stability			
D. Shipment - (From NTP Analytical Contractor or Manufacturer)			
E. Receipt			
F. Storage			
G. Bulk Analyses - Identity and Purity Analyses			
H. Vehicle Analyses			
I. Test Article/Vehicle Method Validation			
J. Test Article/Vehicle 1. Dose Preparation Records 2. Dose Preparation & Room Dose Analyses 3. Homogeneity Study 4. Animal Room Dose Analyses			
K. Inventory/Use Records for Bulk Test Article			
L. Shipment of Test Article Aliquot(s) to NTP Analytical Contractor			
M. Related Correspondence			

NTP Archive Inventory/Index

TEST ARTICLE _____ CAS NO. _____

LABORATORY _____ EXP NO. (C #) _____

ADDRESS _____ NTP STUDY NO._____

CONTRACT NO. _____

SECTION III - RECORDS BY STUDY TYPE

STUDY TYPE _____ ROUTE _____ DATE DOSING INITIATED _____ DATE NECROPSY COMPLETED _____	BOX # or Date Sent	Other Location or Comment	Archive Location
A. NTP SOW (including modifications pertaining to the technical aspects of each study); Laboratory Approved Protocol with Amendments and Deviations B. Vehicle Records Brand, source, dates purchased, lot no(s). dates of use, and test article(s) for which used; for corn oil, peroxide analysis with indication of lot no(s) and dates of analysis C. Dosing Records 1. Dose preparation procedure(s) 2. Dose preparation log 3. Dose Analysis 4. Stability Study D. Diet Analysis E. Water Analysis F. Water Treatment - Commercial and In-house G. Animal Records – Prestudy 1. Species, Strain, Source, Age 2. Receipt 3. Conditions of Quarantine (Caging, food, water) 4. Health Examination/Clinical Signs in Quarantine 5. Release for Study 6. Disposal of Extra Animals			

NTP Archive Inventory/Index

CONTRACT NUMBER _____ LABORATORY _____ TEST ARTICLE _____

SECTION III - RECORDS BY STUDY TYPE	BOX # or Date Sent	Other Location or Comment	Archive Location
H. Animal Records - In-Life, for studies supported by NTP Database 1. Data collection system:In-life & Pathology Protocol Validation Reports (if obtained) 2. Room Location 3. Cage Type and Number per Cage 4. Randomization 5. Identity Code and Confirmation Records 6. Bedding Type, Manufacturer, Contaminants 7. Cage Filter Type and Source 8. Feed Type, Source, Lot No(s), Dates used, Contaminants 9. Type of Water System & Treatment 10. Cage Rotation 11. Rack Rotation 12. Mortality checks 13. In-life Data (Body weight, food and water consumption, clinical signs) a. Data Collection Forms (if used) b. Error Corrections (when applicable) and Exceptions Reports c. Dosing records for gavage studies			
I. Animal Records - In-life, for studies not supported by an NTP data system – Supply the records referred to in H., but maintained in study-specific electronic files, laboratory notebooks and/or forms.			
J. Records for Reproductive and Developmental toxicity parameters: Dam, Litter and Pup Data Including mating, pregnancy, lactation, littering, sex ratio, anogenital distance (AGD), pup body weights, weaning information, correlation of pup id to dam id number, preputial separation, vaginal opening, fetal exams, neurobehavior exams, etc.			
K. Animal Records - Pathology 1. Hard Copies of IANRs (even if electronic used) 2. Slide, block inventory 3. Necropsy Log 3. Organ Weight Records 4. Histology Processing Records and associated records			
L. Animal Room Records 1. Temperature Raw Data 2. Humidity Raw Data 3. Light Cycle/Intensity Measurements			

NTP Archive Inventory/Index

CONTRACT NUMBER _____ LABORATORY _____ TEST ARTICLE _____

SECTION III - RECORDS BY STUDY TYPE	BOX # or Date Sent	Other Location or Comment	Archive Location
4. Air Changes/Air Flow 5. Cleaning Agents Used			
M. Virology Screening Program Data N. Microbiological Testing Reports O. Genetic Monitoring Data (if required)			
P. Special Studies 1. Clinical Lab studies 2. Mammary Tissue Analysis 3. Other special studies			
Q. Final Report 1. Introduction 2. Materials and Methods 3. Results 4. Discussion 5. Appendices			
R. SOPs in Effect for This Study Period including SOPs for data collection system used			
S. Data Collection System For All Phases of Study 1. Type of equipment and software version in use at time of study 2. Processing Logs and All Administrative Records Relevant to Study Data Processing 3. Hardware and Software Receipt and Maintenance Records			
T. Any Internal Computer-Generated Forms/Tables 1. Toxicology 2. Clinical Chemistry 3. Analytical Chemistry 4. Other			
U. Photographs Taken During the Study 1. Gross Observation Documentation 2. Microscopic Observation Documentation 3. Other			
V. Incident (Experimental Impact) Reports			
W. Correspondence			

b) Electronic Submission of Data

All original study documentation and reports shall be organized and submitted electronically, typically on archive-quality CD-R CDs or DVD-R DVDs (or other electronic media approved by the COTR). The usefulness of CDs or DVDs depends on the quality and legibility of the files, as well as the organization of study data and documents. These qualities are the responsibility of the testing facility. The data shall be organized according to the Archive Inventory Index Forms. CDs, DVDs or other readily accessible data transfer media approved by NTP shall be prepared as follows:

a. The electronic files shall also be organized according to the Archive Inventory Index and presented in order on each disc under "View as list". Each file name shall include sufficient information to identify the specific section of the NTP Archive Inventory Index Form to which the data refer. A separate printed copy of the file names shall be included with the disc. If more than one disc is required, the list of file names by section shall clearly indicate on which disc each of the files is located. The entire Archive Inventory Index shall be included at the beginning of the first disc.

b. Portions of data that contain a large data-set may be divided into more than one file to prevent having large files. Each file shall be clearly identified as to its contents.

c. Data are to be scanned at 300 dpi (black and white) to searchable PDF files. The files are to contain document restrictions except for printing and content copying. The files are then burned to discs.

d. All frames of data shall be positioned in the same orientation held for normal reading, i.e. portrait or landscape, whichever is appropriate for that page. Oversized pages shall be reduced or photographed to allow the data to fit a single page. Chart recorded data shall be scrolled left to right or top to bottom depending on how the script runs on the tracings.

e. When data are missing, a blank page with an explanation of the missing data shall be filed at the location of the missing data. If study data records are of such quality that they cannot be scanned, a cross reference shall be included in the file index giving the location of the raw data or records.

f. The original disc shall be examined by the study laboratory for completeness, accuracy, and quality prior to submission to the Archives. Each disc shall contain either a memo with signature or a digital signature (study director) indicating that the PDF is an exact copy of the data. This shall be the first page or on the first page of the first PDF file on each disc.

g. Discs shall be submitted boxed and labeled to prevent mix-up and damage. Each disc must be clearly labeled as to files contained on it. In addition test article, study type and duration, CAS#, C# and laboratory name are to be included on each disc.

h. Once discs are submitted to the Archives, follow-up correspondence or data changes shall be submitted to the Archives with an amended index on additional discs.

BLANK PAGE

XII. REPORT FORMATS

This Section provides example formats and selected required endpoints for Monthly Updates/Progress Reports and for Study Reports.

A. FORMAT AND GUIDELINES FOR MONTHLY PROGRESS REPORT

Cover Page:

Title Page

Monthly Update/Progress Report

Reporting Period

List of Active Study Assignment Numbers

Name and Address of Contract Laboratory

Table of Contents*

* A table of contents shall be provided by the testing laboratory for each Monthly Update/Progress Report. Only those topics that have information to be discussed are to be included in each Monthly Report.

Part 1. **ADMINISTRATION**

a. Status of Cost Proposals

List all study assignments (a) received, but not yet equitably adjusted by signed modification, and (b) those negotiated during the past month. (Note: the Contractor shall not remove an item from this list until negotiations have been completed and the contract amount has been adjusted and reported the previous month.) This table may be modified as needed by the COTR depending on contract needs and requirements. Cost-to-date may be based on current or previous month's cost.

Test Article	Study Assignment Number	Purpose	Date Rec'd By Lab	Date Proposal Submitted	Proposed Amount	Negotiated Amount	Cost-to-Date Status

b. Overall Status of Each Contract

The Contractor shall provide a table as follows:

Study Number	On Cost Target	On Schedule Target
	(yes or no)	(yes or no)

Any "no" responses concerning the cost target shall be explained. The Contractor shall indicate the projected overrun amount and the reason for it.
Any "no" responses concerning the schedule shall be explained and the need for possible contract extension shall be detailed.

c. Other Problems

The Contractor shall briefly discuss any other issues of contract administration not discussed above including any personnel issues such as layoffs affecting personnel critical to NTP studies.

Part 2. **CHEMISTRY**

a. A brief summary of activities performed that month for each test article, i.e. bulk analysis, dose formulation, dose formulation analysis, etc. with summary of the results and discussion of results when they are out of spec.

b. A brief discussion with sufficient supporting documentation to include general problems, reason(s) for problems and approach for resolving and/or preventing them from recurring; deviations from the contract, SOW, protocol, SOPs, and/or milestones. A brief discussion of special or unique scientific findings.

c. Test article Inventory: A listing of test articles shall be provided in each Monthly Progress Report. (See Attachment 1)

Part 3. **LABORATORY ANIMAL MANAGEMENT**

a. Animal Shipments

1) Provide narrative report of incidents on receipt or during quarantine. (Include supplier name/site)
2) Animal Disposition - Submit a completed animal utilization report (See Attachment 2). If animals were used for purposes other than originally designated, indicate at the bottom of the appropriate column.

b. Animal Health

1) Provide narrative report of clinical and/or pathological observations having potential animal health significance. If unique, specify chemical, test phase, species, and sex. List special examinations performed, if any, and provide results if available.

2) Provide a cumulative summary of incidence of health related clinical and/or pathological observations by chemical. The observations could include utero-ovarian infection, preputial gland infections, overgrown incisors, abscesses by region of organ, et cetera each month.

3) Provide a table of serology samples submitted since the last report for murine viral serology including chemical, date bled, species, number of each sex and if quarantine, terminal, sentinel, or moribund animals. If performed, include no. of samples and results of Helicobacter evaluation.

Serology/Diagnostic Samples Submitted

Code	Study	Study phase/ Status	Date	Species	Number (M/F)
yy/yy/yy	ABC	RACB/12 month	9/5/09	Mice	5/5
zz/zz/zz	XYZ	Special/TSAC	9/15/09	Mice	5/5
xx/xx/xx	ZZZ	RACB/TSAC	9/25/09	Rats	5/5

4) Provide a cumulative summary of viral serology profiles by sampling date and age for each chemical each month, including results:

Serology/Diagnostic Results

Rats Study/Phase	Start Date	Test Phase (months)	PVM	Send	RCV/SDA	Parvo	KRV	H-1
ZZZ/Chronic	9/25/09	0	-	-	-	-	NT	NT
		6	-	-	-	-	NT	NT
		12	-	-	-	-	NT	NT
		TS	S	S	S	S	S	S

Mice Study/ Phase	Start Date	Test Phase (months)	PVM	Reo3	GDVII	Ectro	Send	MHV	Parvo	LCM
ABC/ Chronic	3/5/09	0	-	-	-	-	-	-	-	-
		6	-	-	-	-	-	-	-	-
		12	-	-	-	-	-	-	-	-
		18	S	S	S	S	S	S	S	S

- = negative += positive NT = Not tested S = Submitted/results pending

Any positive results, or borderline results that might require retest, are to be discussed in the text.

c. Animal Support

1) Provide narrative report of significant incidents or observations related to animal care.

2) Environmental Control

Provide narrative description of observations that are at variance with NTP specifications and a statement of corrective actions taken.

Provide summary table for all rooms in which test animals are located. (See Attachment 3). Also provide a floor plan designating direction of airflow at each animal room entrance/exit and each air lock, with animal room numbers clearly identified.

d. Training: Provide Table to include attendees, instructor, session title (applicable SOP#), and date.

e. NTP-2000, NIH-07 or other diet information

1) Provide narrative report of incidents in receipt, quality or use of diets.

2) Provide table of current feeding status as follows:

Chemical/Test Phase	Diet/Form	Lot No. and Date of Milling

f. Accreditation/Licensure

1) Describe current status of AAALAC accreditation, NIH assurance, animal care committee, USDA registration, and state and local licenses.

2) Provide date of next accreditation or licensure site visit when known and a narrative statement of the site visit report that pertains to the NTP when the information is available.

Part 4. **TOXICOLOGY**

A brief discussion with sufficient supporting documentation to include general problems and deviations from the contract, SOW, protocol, SOPs, and/or milestones. A brief discussion of relevant and significant scientific findings to include body weight effects, clinical observations, effects on food or water consumption, pregnancy rates, littering results, etc. Provide a table of all animal removals for each test article. If no animals are removed from a study, or for a treatment group within a study, then dose/removal numbers are not needed. (See attachment 4.)

Part 5. **PATHOLOGY**

A brief discussion with sufficient supporting documentation to include general problems and deviations from the contract, SOW, protocol, and/or SOP requirements that affects pathology data, documentation, and/or milestones. A brief discussion of relevant and significant scientific findings to include observance of or change in pathological findings, lesions, survival results, interim sacrifice results, etc.

Part 6. **DATA MANAGEMENT**

A brief discussion with sufficient supporting documentation to include general problem areas relative to the NTP-supplied or in-house systems, system support contractor, hardware maintenance, report production, and deviations from the contract, SOW, protocol, and/or SOP requirements. A brief discussion of unique or special findings.

Attachment 1 Test Article Inventory Status: January 2010

Test Article	Lot/Batch#	Current Inventory	Weekly Usage	Weeks Remaining on Test	Additional Quantity of Test Article Needed for Completion	Approximate Depletion Date of Current Inventory
RAS	8266-F4	3.7 Kg	72 g	23	None	--
DCV	SDC-092179/01	1.9 Kg			Studies Completed	
FN	OF20-M/02	15 Kg [a]	125 g	104	None	
FL	Q112979/01	4.3 Kg	28 g	82	None	--
GBL	600-BLO/01	33.8 Kg	690 g	19	None	--
BEA	005-0120/03	12.2 Kg	610 g	67	30 Kg	5/09
BDCM	250-1/01	0.035 Kg	1.8 g	6	None	
PR 23	UB2158/02	222.1 Kg	11,750 g	3	None	

[a] Not repackaged (approximate figure)

Prepared by: _____ Date: 01/28/10

Attachment 2 MONTHLY ANIMAL UTILIZATION REPORT

Laboratory Identification	1. Test Article		2. Laboratory / 3. Project Officer				3. Principal Investigator		4. Today's Date	
Animal Identification	5. Strain or Stock									
	6. Special Requirements									
	7. Supplier									
	8. Sex									
Animals Received this Month	9. Date Shipped									
	10. Date Received									
	11. Number Shipped									
	12. Number Received									
	13. Boxes Shipped									
	14. Boxes Received									
	Number Discarded on Arrival	15. Dead								
		16. Sick								
	17. Balance (No. Rec'd minus No. Discarded)									
Animals Discarded From Holding Cages During Quarantine This Month	18. Dead									
	19. Sick									
	20. Underweight									
	21. Overweight									
	22. Overage									
	23. Other									
	24 . Total Discarded									
Animals Used This Month	25 . Testing									
	26. Surplus(18+19…+24)									
	27. Total Used (25+26)									

Attachment 3 SUMMARY OF ENVIRONMENTAL CONDITIONS - - May 1, 2004 – May 31, 2004

Room #	Chemical/Phase/Species/Weeks on test	T °F Mean ± SD or TWA ± SD	T Min	T Max	Readings in Spec/ Total Readings	RH% Mean ± SD or TWA ± SD	RH Min	RH Max	Readings in Spec/Total Readings
1	Chemical “ABC”/DRF/R/42	72 ± 1	71	76	718/720	46 ± 3	38	63	717/720
2	Chemical “XYZ”/SC/R&M/6	72 ± 0	71	76	719/720	47 ± 4	38	64	720/720

Attachment 4 ANIMAL REMOVAL SUMMARY - - May 1, 2010 – May 31, 2010

Room Number	Chemical / Phase Dose units	Study week	Dose	Removals Current month / cumulative			
				Rats		Mice	
				Male	Female	Male	Female
5	TFE / F1 ppm	20	0	0/3	1/1	NA	NA
			1	1/2	NA [a]	NA	NA
			2	3/4	7/18	NA	NA
			4	8/13	5/20	NA	NA
			8	NA [a]	7/25	NA	NA
10	APR / DRF mg/Kg	2	10	-- [b]	--	--	--
11	CEB / DRF ppm	10	0	-- [c]	--	--	--
			100	--	--	--	--
			200	--	--	--	--
			400	--	--	--	--
			800	1/1	--	--	--
			1600	3/4	1/1	--	--

Examples above:

[a] NA - No animals treated for this dose group
[b] No animals removed in this study
[c] No animals removed in this dose group

B. GENERIC STUDY REPORT FORMAT

(To be revised upon adoption of a finalized NTP data capture and reporting system under development)

I. ABSTRACT AND SUMMARY TABLE OF SIGNIFICANT TREATMENT-RELATED EFFECTS
(Include only effects considered treatment-related, not merely statistically significant results. Provide enough detail so that the reader can assess the magnitude of the toxicological response)

II. INTRODUCTION
Brief statement (not to exceed 2 pages) to include NTP-supplied information including test article use, production, exposure route, known toxicity and study objectives.

III. MATERIALS AND METHODS

A. CHEMISTRY

1. Test Article
   a. Grade or other test article specific information
   b. Supplier/manufacturer, name and address
   c. Storage conditions for bulk test article
   d. Lots used, giving amount received, date received and dates used for each lot
   e. Test article identify and purity
      (1) Provide information regarding initial bulk analysis, including name of laboratory performing the analyses, methods used, title of report from lab, and results. Clearly identify lot number(s).
      (2) Provide information regarding bulk test article reanalysis, including the number of the SOP used for reanalysis.
      (3) Provide a table of results of the initial and subsequent bulk reanalyses for each lot including dates and analysis results (frozen reference, bulk sample and calculation of relative purity).
2. Vehicle
   a. Vehicle used (feed, water, corn oil, etc.)
   b. Grade/purity/test article specific information
   c. Supplier, Manufacturer/Producer, name and address
   d. Table showing dates analyzed and results of purity analyses; include the number of the SOP used for purity analysis
3. Dose Formulation Procedure
   a. Mixing procedure, including type of container used, duration of mixing, etc. Include the number of the SOP for dose formulation
   b. Stability and homogeneity information on dose formulation (from analytical lab or performed at testing lab)
   c. Storage conditions for dose formulations as appropriate for route of administration, i.e. temperature, humidity, protection from light, maximum length of time stored, etc.
4. Dose Formulation Analysis
   a. Describe the analysis procedure. Include the number of the SOP used for analysis.
   b. Describe the formulation and analysis schedule for both dose preparations and animal room samples.
   c. Provide a table showing dates dose formulations were prepared, dates analyzed, and dates used; and analysis results (mean + S.D.) to include analysis prior to dosing (%high/low of theoretical), and analysis after dosing for unused/stored formulations and animal room samples (%high/low of theoretical & % of original formulation analysis). If formulations were out of spec, indicate if these were remixed or not.

B. ANIMALS AND HUSBANDRY

1. Animals
   a. Species and strain
   b. Source of animals – supplier name and address; date(s) of animal receipt
   c. Examinations to assure health of test animals
   d. Quarantine period
   e. Randomization procedure (to test group, rack, cage)

f. Animal identification procedure
g. Age of animals upon receipt and age and weight of animals when placed on test
h. Serologic and Helicobacter analysis performed including dates samples were taken and results (See format for table of serology results in Monthly Report Format.)

2. Animal Husbandry
a. Describe cages, filters, racks, bedding, feeders, water bottles or automatic waterers used, including the manufacturer's name and address of each item.
b. Describe method of cleaning each item listed above, and where applicable, include the type of washer, cleaning agent, and manufacturer's name and address.
c. Provide schedule for changing cages, filters, racks, bedding, feeders, water bottles, etc.
d. Describe barrier maintenance and disease control procedures. (See Monthly Report for format).
e. Describe room sanitization and pest control procedures.
f. State temperature and humidity ranges, number of room air changes per hour, type of air filtration used, name of filters, manufacturer name and address; light cycle and type of lights used; indicate temperature/humidity excursions, including dates, ranges, number of hours involved.
g. Identify study room(s).
h. Describe number of animals per cage; relationship of control and treated group animals (including rack position); and describe rotation of cages on racks and racks within the room.
i. Identify feed used and storage conditions.
j. Provide source of water supply, any water treatment used and discuss water analyses performed.

C. STUDY DESIGN
1. Design
a. Specify route of administration and frequency of administration.
b. Indicate number of animals per treatment group, number of groups and exposure concentrations.
c. Specify duration of exposure – provide specific dates for first and last treatment.
d. Provide specific details of evaluations performed, such as in-life observations, body weights, reproductive and developmental evaluations, clinical laboratory studies, organ weights, immunotoxicity, neurobehavioral evaluations, etc. Include details of tissue/samples taken, sample collection and handling, analysis method or procedure for conducting special evaluations such as hormone or enzyme analysis, and dates performed.
e. Identify by title all reports submitted under separate cover which are related to these studies, i.e. Prestart Chemistry Reports, Proficiency Reports for Special Studies, etc.
2. In-life Observations and Post-Mortem Examinations
a. Describe in-life observations performed and frequency
b. Describe parental, fetal and offspring data collected and frequency
c. Specific dates for all necropsies
d. Handling of moribund animals
e. Method of euthanasia
f. List of tissues collected at necropsy and processed for histopathological evaluation
g. Histological processes, preservation, embedding, sectioning, staining
h. Statistical analyses, if conducted, to include a short paragraph on methods used

D. SUMMARY TABLE
Provide a summary table to include details of experimental design and animal maintenance such as the example provided below.

IV. RESULTS
This section shall focus on findings that are considered related to administration of the test article. In-text tables can be created to present toxicity findings if appropriate.
For generational studies, data shall be reported separately for each generation and follow a logical pattern similar to the in-life time table for the study. In addition, summary tables shall

present important effects over a, b and c breedings for each generation (if applicable), such as tables presenting number of fertile pairs and related data at each breeding, average litter size per breeding pair for each mating, etc.

Tables/Curves

1. Survival and Mortality Summary by Treatment Group
2. Summary of Body Weight / Body Weight Gain and Body Weight Curves
3. Summary Table for Feed (and Water if a dosed water study) Consumption with Estimate of Test Article Consumed (if applicable)
4. Clinical Observations Summary
5. Summary of Mating, Fertility, Precoital Interval and Gestation Length data
6. Summary of Littering Data including Number of Pups Delivered (Live and Dead)
7. Number of Litters with Live Offspring, Mean Live Litter Size, Sex Ratio
8. Summary of Pup Viability During Lactation, pre and post culling (if applicable)
9. Summary of Pup Observations During Lactation
10. Summary of Pup Weights During Lactation
11. Summary of Anogenital Distance (AGD) measurements
12. Vaginal Opening and Preputial Separation Data, with Wt at Attainment and Weaning
13. Summary Table of Organ Weight and Organ/body Weight Ratios (if applicable)
14. Summary Tables of Clinical Pathology Data (if conducted)
15. Tables of Data from Specific Assessments, including estrus cyclicity, sperm evaluation, behavioral endpoints, etc as appropriate
16. Summary of Gross Pathology Findings
17. Table of Treatment-related Pathology Results

Text shall precede Tables and follow the order of the Tables as presented in the laboratory study report with each section under a separate heading or clustered as appropriate (e.g. “Mortality,” “Body Weight, Body Changes, and Food Consumption,” “Clinical Observations”).

1. Provide a summary of findings for mortality, body weight, food consumption, etc.
2. Describe breeding and littering results as well as any patterns observed for these findings
3. Present results of study-specific evaluations as appropriate
4. Describe pathology findings; if applicable, relate to potential changes in fertility or other reproductive outcomes.

<u>DEVELOPMENTAL/TERATOLOGY STUDIES</u>

Tables/Curves

1. Maternal Mortality/Survival and Disposition Summary
2. Summary of Maternal Body Weight and Body Weight Change Data
3. Summary of Feed (and Water if applicable) Consumption Data
4. Summary of Clinical Observations
5. Summary of Pregnancy Status
6. Summary of Gravid Uterine Wt (if applicable) and Adjusted Maternal Wt
7. Summary of Necropsy Findings
8. Summary of Implantation and Pre/Post-implantation Loss Data
9. Number and Percent Live and Dead Fetuses and Resorptions (early and late)
10. Summary of Fetal Body Weights, by Sex and with Sexes Combined (litter means)
11. Total number and percent of fetuses and litters with any external, soft tissue, or skeletal alteration (malformations and variations), as well as the types and incidences of individual alterations

Text shall precede Tables and follow the order of the Tables as presented in the laboratory study report with each section under a separate heading or clustered as appropriate (e.g. “Mortality,” “Body Weight, Body Changes, and Food Consumption,” “Clinical Observations”).

1. Provide a summary of findings for mortality, pregnancy status, clinical signs, body weight, food consumption, pre and post implantation loss, etc.
2. Describe findings of fetal examinations
3. Present results of study-specific evaluations, as appropriate

V. DISCUSSION AND SUMMARY
1. Provide a discussion of treatment-related findings, including biological significance of the data
2. Describe any problems encountered that would impact interpretation of data
3. Provide a brief summary of salient findings

VI. QUALITY ASSURANCE UNIT STATEMENT
(See Example Below)

VII. APPENDICES
1. Feed: Provide one sample feed label/tag and irradiation certificate
Provide a table that includes lot #, date of milling, and inclusive use dates for feed.
2. Summary of animal room temperature and humidity readings
3. Print-out of pathology report for non-neoplastic findings
4. Individual Animal Data for all non-NTP-data system-collected data, i.e.:
Clinical lab studies (if collected)
Other evaluations, i.e. tissue analysis, hormone analysis, enzyme activity, behavioral assessment, etc.
5. Copy of Contract SOW and Modifications
6. Copy of Laboratory Protocol and Amendments
7. Study Deviation Reports

Example Tables are presented on the following pages. The format of these tables and statistical tests used may change based on output of the NTP data management system currently under development.

TABLE
Summary of Significant Findings
Chemical XYZ: Dose Range-Finding Study for RACB by Gavage

PARAMETERS	DOSE GROUPS[A]				
	0 mg/kg/day	0.1 mg/kg/day	1.0 mg/kg/day	10.0 mg/kg/day	30 mg/kg/day
Male					
Mortality	0/8	0/8	0/8	0/8	1/8
Pre-Breed Body Weight Change (Initial – Week 1)		- 1%	- 2%	- 5%	- 8%
Pre-Breed Body Weight Change (Week 1-2)	-	+ 4%	+ 2%	- 7%	- 12%
Clinical Observations	None observed	None observed	Ruffled Fur 1/8	Ruffled Fur 4/8 Lethargy 4/8	Ruffled Fur 6/8 Lethargy 6/8
Female					
Mortality	0/8	0/8	0/8	0/8	4/8
Pre-Breed Body Weight Change (Initial – Week 1)		- 1%	- 2%	- 4%	- 10%
Pre-Breed Body Weight Change (Week 1-2)	-	+ 4%	+ 2%	- 10%	- 22%
Body Weight Study Day 14					
Body Weight Change (GD0-7)					
Body Weight Change (GD0-21)					
Body Weight Change (PND 0-21)					
Clinical Observations	None observed	None observed	Ruffled Fur 1/10	Ruffled Fur 4/10 Lethargy 4/10	Ruffled Fur 8/10 Lethargy 8/10
Number confirmed mated					
Number pregnant					
Number litters with at least one live pup					
Pre-coital Interval					
Total Number pups/litter					
Number Live pups/litter					
Percent males per Litter					
Mean pup body weight					
Pup Survival (PND 0-4)					
Pup Survival (PND 0-14)					

Materials and Methods for Prenatal Developmental Toxicity Study of Chemical XYZ Administered by Gavage to Harlan Sprague Dawley Rats"

Experimental Design

Size of Test Groups	Core study: 25 time mated females per group Biol samples (test article conc): 4 time mated females per grp
Doses	0, 50, 100, and 200 mg/kg/day by gavage in deionized water
Duration of Dosing	Core study 10/04/10 - 10/19/10 Special study 10/04/10 - 10/16/10
Type and Frequency of Observations	Observed twice daily for moribundity and mortality; body weights recorded daily, food consumption recorded every 3 days, clinical signs recorded daily 2 hours after dosing
Additional Evaluations Conducted	GD 18: Tissue collected for maternal and fetal test article concentration analysis (N = 4)
Caesarean Section	GD 21
Fetal Examinations	All fetuses evaluated for internal, external and skeletal effects; Heads evaluated from 50% of fetuses

Animals and Animal Maintenance

Species	Harlan Sprague Dawley rats
Date Animals received	9/30/10
Animal Source	Harlan Laboratories, Anywhere, USA
Time Held Before Start of Test	4 days
Age/Weight When Placed on Study	11-12 weeks of age (208-235 g)
Method of Animal Distribution	Randomized by stratified body weight
Method of Euthanasia	Dams: Fetuses:
Feed	Irradiated NIH-07 wafers; ABC Inc, Acity, USA Each lot of feed analyzed by ZZZ Labs, City, State
Maximum Storage Time for Feed	120 days post milling
Storage Conditions for Feed	65-73^{o} F; 25-50% RH

Water	Municipal water delivered by an automatic watering system (XYZ System, Anywhere USA); flushed daily; sanitized biweekly
Animals per cage	One
Cages	Polycarbonate cages; Manufacturer and their City/State; changed twice weekly
Cage filters	Spun-bonded polyester filter; XXX #24; Manufacturer and City/State; changed biweekly
Bedding	Irradiated Hardwood Bedding Chips PJ Murphy Forest Products, Corp, Montvale, NJ; changed twice weekly
Racks	Stainless steel; Manufacturer and City/State; changed biweekly
Cage Washer	Tunnel type (4 cycles); Manufacturer and City/State
Rack Washer	Cabinet type (2 cycles); Manufacturer and City/State
Cage & Rack Washing Compound	Brand, Manufacturer and City/State.
Animal Room Environment	Actual range 69-76 ^{o} F; 2,502/2,520 readings within Spec Actual range 35-69 % RH; 2,495/2,520 readings within Spec 12 hours of fluorescent light per day; 10 - 12 room air changes per hour
Room Air Filter	Fiberglass roughing filter; Manufacturer and City/State changed biweekly

Test Article Vehicle Mixture

Mixture Preparation	Weighed portion of Chemical "XYZ" and mixed with Deionized water to make up selected doses Mixture stirred for 15 minutes with electric stir bar.
Maximum Storage Time	35 days
Storage Conditions	Stored in airtight, amber glass bottles, protected from light, Ambient conditions (72 $\pm$ 3^{o} F),

EXAMPLE TABLE
Summary of Male and Female Mortality Data
Chemical XYZ: Dose Range-Finding Study for RACB

PARAMETERS	DOSE GROUPS[a]				
	0 mg/kg/day	0.1 mg/kg/day	1.0 mg/kg/day	3.0 mg/kg/day	10 mg/kg/day
Male					
Found Dead	0/8	0/8	0/8	0/8	2/8
Moribund Euthanasia	0/8	0/8	0/8	0/8	0/8
Euthanized at Study Termination	8/8	8/8	8/8	8/8	6/8
Female					
Found Dead	0/8	0/8	0/8	0/8	2/8
Moribund Euthanasia	0/8	0/8	0/8	0/8	0/8
Euthanized at Study Termination	8/8	8/8	8/8	8/8	6/8

[a] Number of animals dead, moribund, or euthanized at study termination / total number of animals in a group

EXAMPLE TABLE
SUMMARY OF BODY WEIGHTS (GRAMS)
Chemical XYZ: Dose Range-Finding Study for RACB

DAY OF STUDY	DOSE GROUPS[a]					
	0 mg/kg/day	0.1 mg/kg/day	1.0 mg/kg/day	3.0 mg/kg/day	10 mg/kg/day	TREND[b]
Males						
1	378.9 ± 6.51 (8)	379.0 ± 4.88 (8)	378.0 ± 3.29 (8)	381.1 ± 6.81 (8)	379.6 ± 6.29 (8)	P=0.721
4	413.4 ± 7.82 (8)	399.1 ± 4.29 (8)	409.5 ± 4.66 (8)	398.3 ± 13.16 (8)	408.0 ± 9.02 (8)	P=0.830
7	435.5 ± 8.40 (8)	422.4 ± 5.62 (8)	428.9 ± 5.92 (8)	431.4± 10.3 (8)	426.5 ± 9.95 (8)	P=0.868
11	467.0 ± 9.37 (8)	461.7 ± 5.65 (8)	459.0 ± 7.36 (8)	464.4 ± 12.13 (8)	456.2 ± 10.55 (8)	P=0.849
14	495.8 ± 11.04 (8)	491.7 ± 5.98 (8)	484.5 ± 9.23 (8)	488.4 ± 12.25 (8)	480.4 ± 12.73 (6)	P=0.651
Female						
1	244.6 ± 3.83 (8)	242.2 ± 5.35 (8)	247.3 ± 3.85 (8)	239.7 ± 3.56 (8)	244.2 ± 3.05 (8)	P=0.972
4	246.8 ± 4.27 (8)	250.7 ± 9.02 (8)	248.3 ± 3.54 (8)	244.4 ± 12.22 (8)	247.6 ± 4.05 (8)	P=0.991
7	275.5 ± 4.86 (8)	279.0 ± 9.30 (8)	269.5 ± 5.60 (8)	266.5 ± 10.11 (8)	270.3 ± 5.11 (8)	P=0.174
11	300.9 ± 4.68 (8)	309.4 ± 10.41 (8)	295.5 ± 5.62 (8)	294.4 ± 6.88 (8)	289.3 ± 6.96 (8)	P=0.124
14	357.1 ± 12.39 (8)	376.0 ± 14.05 (8)	353.2 ± 6.88 (8)	357.6 ± 11.24 (8)	339.4 ± 12.06 (6)	P=0.253

a Mean ± standard error (number of animals)
b Each dose group is compared to the control with Shirley's test when a trend is present (p=0.01 from Jonckheere's trend test) otherwise Dunn's test is applied (* = p<0.05)
(This table is provided as an example only. Note that statistical tests required for actual studies and table format may change based on the development of an NTP data management system)

EXAMPLE TABLE
SUMMARY OF DAILY FEED CONSUMPTION (G/ANIMAL/DAY)
Chemical XYZ: Dose Range-Finding Study for RACB

DAY OF STUDY	DOSE GROUPS [a]					
	0 mg/kg/day	0.1 mg/kg/day	1.0 mg/kg/day	3.0 mg/kg/day	10 .0 mg/kg/day	TREND[b]
Male						
1-3	26.4 ± 11.06 (8)	23.6 ± 1.67 (8)	23.9 ± 0.56 (8)	26.2 ± 0.97 (8)	25.0 ± 1.30 (8)	P=0.896
4-7	27.0 ± 1.06 (8)	24.6 ± 3.07 (8)	25.3 ± 0.67 (8)	22.2 ± 2.26 (8)	25.4 ± 1.01 (8)	P=0.093
Female						
1-3	13.8 ± 0.77 (8)	16.6 ± 0.85 (8)	15.1 ± 0.45 (8)	14.9 ± 0.44 (8)	14.6 ± 1.62 (8)	P=0.365
4-7	17.0 ± 0.06 (8)	18.6 ± 0.93 (8)	17.9 ± 0.40 (8)	17.8 ± 0.06 (8)	17.1 ± 1.13 (8)	P=0.511
Male and Female						
8-10	19.3 ± 0.74 (8)	19.0 ± 1.11 (8)	19.3 ± 1.01 (8)	19.2 ± 0.48 (8)	17.7 ± 0.74 (8)	P=0.0146
11-14	22.8 ± 0.54 (8)	22.3 ± 0.88 (8)	*19.6 ± 0.81 (8)	*20.7 ± 0.44 (8)	*20.8 ± 1.56 (8)	P=0.005
15-17	22.5 ±0.43 (8)	19.5 ± 2.31 (8)	*20.5 ± 0.48 (8)	20.0 ± 0.99 (8)	*17.8 ± 1.66 (8)	P=0.002
18-21	23.5 ± 0.62 (8)	24.0 ± 0.72 (8)	22.2 ± 0.51 (8)	22.8 ± 0.73 (8)	*20.5 ± 0.99 (8)	P=0.009
22-24	21.1± 0.95 (8)	20.9 ± 0.49 (8)	20.0 ± 0.59 (8)	20.7 ± 0.54 (8)	19.4 ± 0.82 (8)	P=0.035
25-29	23.8 ± 0.66 (8)	23.9 ± 0.57 (8)	22.9 ± 0.72 (8)	23.6 ± 0.60 (8)	21.8 ± 0.98 (8)	P=0.102

a Mean ± standard error (number of animals)
b Each dose group is compared to the control with Shirley's test when a trend is present ($p<0.01$ from Jonckheere's trend test), otherwise Dunn's test is applied (* = $p<0.05$).
(This table is provided as an example only. Note that statistical tests required for actual studies and table format may change based on the development of an NTP data management system)

For studies where the test article is administered via diet, a similar table is required to show test article consumed.

EXAMPLE TABLE
SUMMARY OF REPRODUCTIVE PERFORMANCE
Chemical XYZ: Dose Range-Finding Study for RACB

PARAMETER	DOSE GROUPS[a]					
	0 mg/kg/day	0.1 mg/kg/day	1.0 mg/kg/day	3.0 mg/kg/day	10 mg/kg/day	TREND
No. Females Paired	8	8	8	8	8	
Mating Index b,d,e	6/8 (75%)	8/8 (100)	8/8 (100)	8/8 (100)	7/8 (88)	P=0.21
Fertility Index b,d,e	6/8 (75%)	8/8 (100)	8/8 (100)	8/8 (100)	7/8 (88)	P=0.21
Fecundity b,d,e	6/6 (100%)	8/8 (100)	8/8 (100)	8/8 (100)	7/7 (100)	P=0.21
Live Pups per Litter c,f,g						
Male	7.0 ± 1.06 (6)	8.8 ±1.19 (8)	6.4 ± 0.78 (8)	6.6 ± 0.32 (8)	4.6 ± 0.95 (7)	P=0.016
Female	5.8 ± 0.75 (6)	7.0 ± 0.85 (8)	6.8 ± 1.03 (8)	7.9 ±0.35 (8)	7.4 ± 0.75 (7)	P=0.036
Combined	12.8 ± 0.40 (6)	15.8 ± 1.05 (8)	13.1 ± 1.16 (8)	14.5 ± 0.27 (8)	12.0 ± 1.48 (7)	P=0.860
Proportion Pups Born Alive c,f,g	0.98 ± 0.02 (6)	1.00 ± 0.00 (8)	0.96 ± 0.02 (8)	0.98 ± 0.01 (8)	0.98 ± 0.02 (7)	P=0.915
Sex of Pups Born Alive: Males/Total c,g,h	0.54 ± 0.07 (6)	0.56 ± 0.05 (8)	0.50 ± 0.05 (8)	0.46 ± 0.02 (8)	0.36 ± 0.05 (7) *	P=0.002
Live Pup Wt (g) c,g,h						
Male	6.60 ± 0.19 (6)	6.33 ± 0.23 (8)	6.50 ± 0.17 (8)	6.38 ± 0.14 (8)	6.89 ± 0.23 (7)	P=0.399
Female	6.20 ± 0 15 (6)	6.06 ± 0.10 (8)	6.18 ± 0.14 (8)	6.04 ± 0.13 (8)	6.41 ± 0.16 (7)	P=0.471
Combined	6.39 ± 0.15 (6)	6.24 ± 0.14 (8)	6.35 ± 0.17 (8)	6.19 ± 0.14 (8)	6.57 ± 0 17 (7)	P=0.503
Anogenital Distance (mm) g, i						
Male	2.70 ± 0.10 (6)	2.59 ± 0.06 (8)	2.55 ± 0.05 (8)	2.49 ± 0.06 (8)	2.55 ± 0.05 (7)	P=0.012
Female	1.31 ± 0.06 (6)	1.25 ± 0.05 (8)	1.24 ± 0.05 (8)	1.29 ± 0.04 (8)	1.22 ± 0.05 (7)	P=0.511
Average Days to Litter c,g,i	24.7 ± 0.42 (6)	24.8 ± 0.56 (8)	25.0 ± 0.42 (8)	24.8 ± 0.41 (8)	25.4 ± 0.30 (7)	P=0.293

a Statistical significance for comparisons of dosed groups to controls: * = p<0.05.
b Only those pairs surviving cohabitation were included for statistical analysis of data.
c Only those females surviving to the end of the parturition period were included for statistical analysis of the data.
d Mating index: Number of confirmed mated females/ number of cohabiting pairs (percent)
Fertility Index: Number pregnant / number of cohabitating pairs (percent)
Fecundity: Number with live litters / number pregnant (percent)
e P=value from Cochran-Armitage trend test. Dosed groups compared to control group by Chi-square test. If fertility index > 1, then it is set equal to 1 for statistical analysis.
f Mean ± standard error (number of pregnant pairs).
g Each dose group is compared to the control group by Shirley's test if P<0.01 from Jonckheere's trend test. Otherwise Dunn's test is applied.
h Mean ± standard error (number of pregnant pairs producing live pups).
i Mean ± standard error (number of dams)
(This table is provided as an example only. Note that statistical tests required for actual studies and table format may change based on the development of an NTP data management system)

EXAMPLE TABLE
SUMMARY OF LITTERING INDEX OF BREEDING PAIRS
Chemical XYZ: Reproductive Assessment by Continuous Breeding when Administered to Harlan Sprague Dawley Rats by Gavage

LITTER	DOSE GROUPS[a]				
	0 mg/kg/day	1 mg/kg/day	3 mg/kg/day	10 mg/kg/day	TREND[b]
F1a	19/20 (95)	19/20 (95)	20/20 (100)	20/20 (100)	P= 0.323
F1b	17/20 (89)	19/20 (95)	19/19 (100)	16/19 (84)	P=0.067
F1c	13/19 (68)	15/19 (79)	17/19 (89)	13/17 (76)	P=0.491
F2a					
F2b					
F2c					

[a] Number of females delivering / number of cohabitating pairs (percent pregnant).
[b] P-value from Cochran-Armitage trend test for decrease in pregnancies.
Each dose group is compared to the control group with a chi-square test (* = P<0.05).
(This table is provided as an example only. Note that statistical tests required for actual studies and table format may change based on the development of an NTP data management system)

EXAMPLE TABLE
F1 - SUMMARY OF REPRODUCTIVE PERFORMANCE OF COHABITED PAIRS DURING CONTINUOUS BREEDING
Chemical XYZ: Reproductive Assessment by Continuous Breeding when Administered to Harlan Sprague Dawley Rats by Gavage

PARAMETER	DOSE GROUPS[a]				
	0 mg/kg/day	1.0 mg/kg/day	3.0 mg/kg/day	10 mg/kg/day	TREND
No. Females Paired	20	20	20	20	
Average Litters per Pair b,d	2.5 0 ±.02 (20)	2.7 ±.0.17 (20)	2.8 ±.0.12 (20)	2.5 ±.0.18 (20)	P=0.865
Live Pups per Litter c,d					
Male	6.1 ± 0.39 (19)	5.2 ± 0.78 (19)	*3.9 ± 0.32 (20)	*3.1 ± 0.95 (20)	P=0.001
Female	6.3 ± 0.75 (19)	*5.1 ± 1.03 (19)	*4.7 ± 0.35 (20)	*3.4 ± 0.75 (20)	P=0.001
Combined	12.4 ± 0.40 (19)	*10.4 ± 1.16 (19)	*8.2 ± 0.27 (20)	*6.5 ± 1.48 (20)	P=0.001
Proportion Pups Born Alive c,d	0.97 ± 0.016 (19)	0.96 ± 0.015 (19)	0.97 ± 0.011 (20)	0.89 ± 0.016 (20)	P=0.007
Sex Pups Born Alive (Males/Total) d,e	0.49 ± 0.070 (19)	0.50 ± 0.051 (19)	0.46 ± 0.022 (20)	0.46 ± 0.047 (20)	P=0.466
Live Pup Wt (g) d,e					
Male	6.52 ± 0.188 (19)	6.46 ± 0.168 (19)	6.46 ± 0.135 (20)	*6.11 ± 0.230 (20)	P=0.001
Female	6.15 ± 0 148 (19)	6.18 ± 0.140 (19)	6.16 ± 0.133 (20)	*6.01 ± 0.159 (20)	P=0.013
Combined	6.35 ± 0.150 (19)	6.32 ± 0.165 (19)	6.32 ± 0.135 (20)	*6.10 ± 0 172 (20)	P=0.001
Live Pup Wt (g) Adjusted for Litter Size f,g					
Male	6.60 ± 0.188 (19)	6.50 ± 0.168 (19)	6.38 ± 0.135 (20)	*6.13 ± 0.230 (20)	P=0.001
Female	6.20 ± 0 148 (19)	6.18 ± 0.140 (19)	6.04 ± 0.133 (20)	*6.00 ± 0.159 (20)	P=0.001
Combined	6.39 ± 0.150 (19)	6.35 ± 0.165 (19)	6.19 ± 0.135 (20)	*6.12 ± 0 172 (20)	P=0.001

a Statistical significance for comparisons of dosed groups to controls: * = p<0.05.
b Mean ±. Standard error (Number of cohabitated pairs).
c Mean ±. Standard error (Number of pregnant pairs).
d Each dose group is compared to the control group with Shirley's test when a trend is present (P<0.01) from Jonckheere's trend test), otherwise, Dunn's test is used (* == P<0.05).
e Mean ±. Standard error (number of fertile pairs producing live pups).
f Least squares estimate of the mean of the average pup weight from each fertile pair, adjusted for average litter size ±. Standard error (number of fertile pairs producing live pups)
g Overall P-values from an F-test for equality of group means. Dunnett's test was used to compare each dosed group to the control group.
(This table is provided as an example only. Note that statistical tests required for actual studies and table format may change based on the development of an NTP data management system)

EXAMPLE TABLE
SUMMARY OF REPRODUCTIVE PERFORMANCE DURING F1a LITTER
Chemical XYZ: Reproductive Assessment by Continuous Breeding when Administered to Harlan Sprague Dawley Rats by Gavage

PARAMETER	DOSE GROUPS[a]				
	0 mg/kg/day	1.0 mg/kg/day	3.0 mg/kg/day	10 mg/kg/day	TREND
No. Females Paired					
No. Females Delivering/No. Females Paired b,d,e	19/20 (95)	19/20 (95)	20/20 (100)	20/20 (100)	P=0.323
Live Pups / Litter c,f,g					
Male	6.9 ± 0.39 (19)	6.6 ± 0.78 (19)	6.2 ± 0.32 (20)	*4.5 ± 0.95 (20)	P=0.001
Female	7.3 ± 0.75 (19)	6.5 ± 1.03 (19)	6.7 ± 0.35 (20)	*4.7 ± 0.75 (20)	P=0.008
Combined	14.2 ± 0.40 (19)	13.3 ± 1.16 (19)	12.9 ± 0.27 (20)	*9.2 ± 1.48 (20)	P=0.001
Prop. Pups Born Alive c,f,g	0.99 ± 0.016 (19)	0.96 ± 0.015 (19)	0.99 ± 0.011 (20)	*0.91 ± 0.016 (20)	P=0.005
Sex Pups Born Alive (Males/Total) c,h,g	0.49 ± 0.070 (19)	0.50 ± 0.051 (19)	0.46 ± 0.022 (20)	0.46 ± 0.047 (20)	P=0.466
Live Pup Wt (g) c,g,h					
Male	6.52 ± 0.188 (19)	6.46 ± 0.168 (19)	6.46 ± 0.135 (20)	*6.11 ± 0.230 (20)	P=0.001
Female	6.15 ± 0 148 (19)	6.18 ± 0.140 (19)	6.16 ± 0.133 (20)	*6.01 ± 0.159 (20)	P=0.013
Combined	6.35 ± 0.150 (19)	6.32 ± 0.165 (19)	6.32 ± 0.135 (20)	*6.10 ± 0 172 (20)	P=0.001
Live Pup Wt (g) Adjusted for Litter Size f,g					
Male	6.60 ± 0.188 (19)	6.50 ± 0.168 (19)	6.38 ± 0.135 (20)	*6.13 ± 0.230 (20)	P=0.001
Female	6.20 ± 0 148 (19)	6.18 ± 0.140 (19)	6.04 ± 0.133 (20)	*6.00 ± 0.159 (20)	P=0.001
Combined	6.39 ± 0.150 (19)	6.35 ± 0.165 (19)	6.19 ± 0.135 (20)	*6.12 ± 0 172 (20)	P=0.001
AGD (mm) k,g					
Male	2.65 ± 0.106	2.55 ± 0.042	2.64 ± 0.054	2.61 ± 0.062	P=0.996
Female	1.32 ± 0.025	1.29 ± 0.022	1.32 ± 0.029	1.35 ± 0.031	P=0.466
Ave Days to Litter c,k,g	25.1 ± 0.77	24.6 ± 0.29	24.6 ± 0.24	25.2 ± 0.36	P-0.116
Pup Survival g,l					
Male PND 4	1.00 ± 0.000 (19)	0.99 ± 0.011 (19)	1.00 ± 0.00 (20)	* 0.85 ± 0.101	P=0.041
Female PND 4	1.00 ± 0.000 (19)	1.00 ± 0.000 (19)	0.92 ± 0.077 (19)	0.91 ± 0.091	P=0.167
Combined PND 4	1.00 ± 0.000 (19)	0.86 ± 0.090 (19)	0.093 ± 0.011	0.082 ± 0.102	P=0.085

a Statistical significance for comparisons of dosed groups to controls: * = $p<0.05$.
b Only those pairs surviving cohabitation were included for statistical analysis of data.
c Only those females surviving to the end of the parturition period were included for statistical analysis of the data.
d (percent females delivering)
e P=value from Cochran-Armitage trend test. Dosed groups compared to control group by Chi-square test. If fertility index > 1, then it is set equal to 1 for statistical analysis.
f Mean ± standard error (number of pregnant pairs).
g Each dose group is compared to the control group by Shirley's test if $P<0.01$ from Jonckheere's trend test. Otherwise Dunn's test is applied.
h Mean ± standard error (number of pregnant pairs producing live pups).
i Least squares estimate of mean pup weight adjusted for average litter size ± standard error (number of pregnant pairs producing live pups).
j Overall differences tested with an F-test, pairwise comparisons from Dunnett's test.
k Mean ± standard error.
l Mean of (number of live pups/number of pups born alive) ± standard error (number of litters). Ratios greater than 1 or increases in survival over time indicate mis-sexing of pups.
(This table is provided as an example only. Note that statistical tests required for actual studies and table format may change based on the development of an NTP data management system)

EXAMPLE TABLE
SUMMARY OF F2a PUP DEFECTS (PRENATAL DEVELOMENTAL TOXICITY COHORT)
Chemical XYZ: Reproductive Assessment Following Administration to Harlan Sprague Dawley Rats by Gavage

PARAMETER	Group (Dose Level in mg/kg/day)			
	1 (0)	2 (1.0)	3 (3.0)	4 (10.0)
Total number of pups examined	272	264	260	199
Total number of litters examined	19	19	20	20
EXTERNAL VARIATIONS AND MALFORMATIONS				
Number of pups examined	272	264	260	199
Number of pups showing Variation	0	0	0	0
% of pups showing Variation	0.0	0.0	0.0	0.0
Number of litters affected	0	0	0	0
Number of pups showing Malformation	0	0	0	2
% of pups showing Malformation	0.0	0.0	0.0	1.0
Number of litters affected	0	0	0	2
VISCERAL DEFECTS				
Number of pups examined	270	260	260	199
Number showing Variation	3	1	2	7
% of pups showing Variation	1.1	0.08	0.75	3.1
Number of litters affected	2	1	2	5
Number showing Malformation	0	0	1	1
% of pups showing Malformation	0.0	0.0	0.3	0.3
Number of litters affected	0	0	1	1
BOUIN'S HEAD DEFECTS				
Number of pups examined	143	121	135	107
Number showing Variation	0	0	0	0
% of pups showing Variation	0.0	0.0	0.0	0.0
Number of litters affected	0	0	0	0
Number showing Malformation	0	0	0	0
% of pups showing Malformation	0.0	0.0	0.0	0.0
Number of litters affected	0	0	0	0
SKELETAL DEFECTS				
Number of pups examined	272	264	260	199
Number showing Variation	12	13	9	19
% of pups showing Variation	4.6	5.1	3.5	10.4
Number of litters affected	8	8	7	7
Number showing Malformation	0	0	1	0
% of pups showing Malformation	0.0	0.0	0.4	0.0
Number of litters affected	0	0	1	0

(This table is provided as an example only. Note that statistical tests required for actual studies and table format may change based on the development of an NTP data management system)

EXAMPLE TABLE
SUMMARY OF F1c WEANLING SEXUAL DEVELOPMENT DATA
Chemical XYZ: Reproductive Assessment Following Administration to Harlan Sprague Dawley Rats by Gavage

PARAMETERS	DOSE GROUPS[a]				
	0 mg/kg/day	1.0 mg/kg/day	3.0 mg/kg/day	10.0 mg/kg/day	TREND[b,c,d]
Males					
Day of Prepuce Separation	42.8 ± 0.29 (120)	42.1 ± 0.32 (119)	44.0 ± 0.24 (118)	46.4 ± 0.76 (110)	P<0.001[b,c]
BW at Prepuce Separation	200 ± 0.29 (120)	201 ± 0.29 (120)	203 ± 0.29 (120)	204 ± 0.29 (120)	P=0.905
Day of Testicular Descent	29.0 ± 0.23 (120)	29.0 ± 0.22 (119)	29.5 ± 0.28 (118)	* 31.5 ± 0.72 (110)	P<0.001[b,c]
BW at Testicular Descent	110 ± 0.29 (120)	100 ± 0.29 (120)	109 ± 0.29 (120)	108 ± 0.29 (120)	P=0.905
BW at Weaning	100 ± 0.29 (120)	100 ± 0.29 (120)	100 ± 0.29 (120)	100 ± 0.29 (120)	P=0.905
Females					
Day of Vaginal Opening	33.1 ± 0.29 (120)	32.7 ± 0.56 (120)	33.8 ± 0.30 (120)	* 35.2 ± 0.71(120)	P=0.004[b,c]
BW at Attainment of Vag. Opening	110 ± 0.29 (120)	110 ± 0.29 (120)	110 ± 0.29 (120)	110 ± 0.29 (120)	P=0.905
BW at Weaning	100 ± 0.29 (120)	100 ± 0.29 (120)	100 ± 0.29 (120)	100 ± 0.29 (120)	P=0.905

[a] Mean standard error (number of animals)
[b] Each dose group is compared to the control with Shirley's test when a trend is present (P<0.01 from Jonckheere's trend test), otherwise Dunn's test is applied (* = P<0.05)
[c] covaried by body weight at day of attainment
[d] covaried by body weight at day of weaning
(This table is provided as an example only. Note that statistical tests required for actual studies and table format may change based on the development of an NTP data management system)

EXAMPLE TABLE

Mean Organ Weights and Percent Organ-to-Body Weight for Males
For the ABC Study of Chemical "XYZ" in Harlan Sprague Dawley Rats (Timepoint xyz).

Treatment Groups (mg/Kg)	Terminal Body Wt (g) (n)	Liver		Lung		Adrenal		Thymus		Testis, Right	
		Absolute Wt. (g)	Relative Wt. [a]	Absolute Wt. (g)	Relative Wt.	Absolute Wt. (g)	Relative Wt.	Absolute Wt. (g)	Relative Wt.	Absolute Wt. (g)	Relative Wt.
0	354.2 ± 18.1 (10)	13.201 ± 0.906	3.725 ± 0.113	1.670 ± 0.227	0.471 ± 0.053	0.0747 ± 0.003	0.022 ± 0.011	0.306 ± 0.057	0.086 ± 0.014	1.506 ± 0.045	0.426 ± 0.015
1000	364.6 ± 26.4 (10)	13.201 ± 0.906	3.801 ± 0.182	1.710 ± 0.293	0.468 ± 0.062	0.0791 ± 0.003	0.0.23 ± 0.018	0.291 ± 0.032	0.080 ± 0.011	1.534 ± 0.078	0.421 ± 0.019
2000	351.3 ± 14.1 (10)	13.201 ± 0.906	3.712 ± 0.126	1.773 ± 0.182	0.505 ± 0.047	0.0798 ± 0.003	0.0.22 ± 0.011	0.311 ± 0.036	0.088 ± 0.009	1.498 ± 0.112	0.426 ± 0.033
3000	345.4 ± 14.5 (10)	13.201 ± 0.906	3.789 ± 0.135	1.648 ± 0.189	0.477 ± 0.050	0.0786 ± 0.006	0.024 ± 0.013	0.302 ± 0.042	0.087 ± 0.012	1.500 ± 0.055	0.435 ± 0.017
4000	353.9 ± 19.8 (10)	13.201 ± 0.906	3.850 ± 0.211	1.673 ± 0.205	0.472 ± 0.053	0.0810 ± 0.006	0.0.026 * ± 0.013	0.310 ± 0.051	0.084 ± 0.015	1.522 ± 0.073	0.431 ± 0.023
5000	339.2 ± 24.0 (10)	13.201 ± 0.906	3.986 ** ± 0.129	1.609 ± 0.178	0.475 ± 0.036	0.0841 ± 0.009	0.027 ** ± 0.022	0.292 ± 0.038	0.086 ± 0.009	1.395 ± 0.305	0.402 ± 0.111

[a] Relative weight = mg organ weight/g B.W. (mean ± SD)

* $p \leq 0.05$

** $p \leq 0.01$

(This table is provided as an example only. Note that statistical tests required for actual studies and table format may change based on the development of an NTP data management system)

Clinical Chemistry Results for Female Rats in the Gavage Toxicity Study of Chemical XYZ
(Mean ± S.D., N = 10)

EXAMPLE TABLE
Day 22

Treatment Groups (mg/Kg)	Albumin g/dl	Protein g/dl	CK u/L	Creatinine mg/dl	ALP u/L	ALT u/L	SDH u/L	Bile Acid umol/L	BUN mg/dl	GGT u/L	Glucose mg/dL
0	4.4 ± 0.2	6.3 ± 0.2	268.1 ± 15.6	0.72 ± 0.04	431 ± 22	45.1 ± 5.2	20.2 ± 2.2	31.5 ± 4.3	12.3 ± 0.9	1.2 ± 0.4	139 ± 5
250	4.4 ± 0.1	6.3 ± 0.2	270.8 ± 15.1	0.73 ± 0.04	435 ± 20	48.3 ± 7.1	22.1 ± 3.1	34.2 ± 3.0	12.4 ± 0.8	1.4 ± 0.6	142 ± 7
500	4.3 ± 0.2	6.5 ± 0.2	289.4 ± 12.3	0.75 ± 0.03	442 ± 32	43.6 ± 8.9	25.0 ± 5.1	34.6 ± 2.9	13.0 ± 0.8	1.3 ± 0.5	141 ± 6
1000	4.3 ± 0.1	6.4 ± 0.1	285.2 ± 13.6	0.7 3± 0.02	425 ± 15	46.1 ± 7.1	23.2 ± 3.4	30.2 ± 4.6	12.0 ± 0.9	1.5 ± 0.5	136 ± 10
2000	4.4 ± 0.2	6.3 ± 0.1	292.6 ± 28.5	0.74 ± 0.02	446 ± 43	45.7 ± 10.3	24.4 ± 4.6	33.8 ± 4.0	11.8 ± 0.7	1.7 ± 0.7	140 ± 6
4000	4.2 ± 0.2	6.4 ± 0.2	278.8 ± 19.5	0.75 ± 0.05	442 ± 48	72.2 ± 12.1 **	26.1 ± 7.1	44.1 ± 3.3 **	12.7 ± 1.1	3.2 ± 1.1 *	143 ± 7

* $p \leq 0.05$
** $p \leq 0.01$

NOTE: Table is to include results for each time point samples were analyzed. Similar table is to be included for hematology parameters
(This table is provided as an example only. Note that statistical tests required for actual studies and table format may change based on the development of an NTP data management system)

Treatment-related Lesions in Female Rats in the Study of Chemical "XYZ" (Timepoint ABC)

EXAMPLE TABLE

Organ/Diagnosis	**Incidence of Lesions (Mean Severity Treatment Groups (ppm)**					
Dose	0	8	16	32	62	125
KIDNEY	10 [a]	NE	NE	10	10	10
Inflammation	0 [b]	--	--	0	3 (1.3) [c]	10 (1.6)
Tubular epithelium, necrosis	0	--	--	0	0	2 (2.0)

[a] Number of tissues or animals examined

[b] Number of diagnoses made

[c] Severity: 1 = minimal; 2 = mild; 3 = moderate; 4 = marked

NE = Not examined

(This table is provided as an example only. Note that statistical tests required for actual studies and table format may change based on the development of an NTP data management system)

Dam and Litter Data for the Perinatal Toxicity Dose Range finding Study of Chemical X Administered via Drinking Water in Harlan Sprague Dawley Rats
(It is anticipated that this table format will change based upon output from the NTP data collection and management system under development)

SAMPLE TABLE

		Concentration					
		0 mg/L	125 mg/L	250 mg/L	500 mg/L	1000 mg/L	2000 mg/L
Number of Time-Mated Females on GD6		8	8	8	8	8	8
Number of Pregnant Females		7	8	8	6	6	6
Number of Dams with Litters on Postnatal Day (PND) 0		6	8	7	6	6	6
Number of Females Non-Pregnant		1	0	0	2	2	2
Number of Dams Not Delivering With Resorptions/Fetuses		1	0	1[a]	0	0	0
PND 1	**Number of Litters**	6	8	7	6	6	6
	Mean Litter Size[b]	11.0	12.8	12.0	12.0	10.7	12.0
	Mean Number (%) of Male Pups/Litter	4.8 (44)	5.6 (44)	5.9 (49)	6.5 (54)	4.8 (45)	6.8 (57)
	Mean Number (%) of Female Pups/Litter	6.2 (56)	7.1 (56)	6.1 (51)	5.5 (46)	5.8 (55)	5.2 (43)
	Mean Body Weight of Male Pups/Litter (g)	7.85	7.57	7.56	7.30	8.06	6.84
	Mean Body Weight of Female Pups/Litter (g)	7.24	7.10	7.40	7.11	6.67	6.44
PND 4	**Number of Litters**	6	8	7	6	6	6
	Mean Litter Size	11.0	12.8	12.0	12.0	10.7	12.0
	Mean Number (%) of Male Pups/Litter	4.8(44)	5.6 (44)	5.9 (49)	6.5 (54)	4.8 (45)	6.8 (57)
	Mean Number (%) of Female Pups/Litter	6.2(56)	7.1 (56)	6.1 (51)	5.5 (46)	5.8 (55)	5.2 (43)
	Mean Body Weight of Male Pups/Litter	9.85	9.57	9.56	7.40	9.98	8.86
	Mean Body Weight of Female Pups/Litter	9.24	9.10	9.54	9.12	7.78	8.44

a. One dam was terminated on GD24 due to incomplete labor.
b. Only live pups on their respective PND were included for all calculations.

(This table is provided as an example only. Note that statistical tests required for actual studies and table format may change based on the development of an NTP data management system)

Dam Status, Pup Status, and Pup Number Cross-reference List for the Dosed-water Perinatal Toxicity Study of XYZ in Harlan Sprague Dawley Rats
Note: dams were mated 1:1 (1 male to 1 female) at the vendor (This table format may change based on output from the NTP data management System under development)

SAMPLE TABLE

Concentration	Dam ID	Parturition Date	Pre-Wean Pup Number	Pup ID	Sex	Post-Wean Study ID	Disposition
2000 mg/L	601	6/8/09	1	6011	Male		
			2	6012	Male	54	
			3	6013	Male	59	
			4	6014	Male		
			5	6015	Female	111	
			6	6016	Female	112	
			7	6017	Female	114	
			8	6018	Female		
2000 mg/L	602	6/8/09	1	6021	Male	56	
			2	6022	Male	60	
			3	6023	Male		
			4	6024	Male	55	
			5	6025	Male		
			6	6026	Male		
			7	6027	Female	120	
			8	6028	Female	118	
2000 mg/L	603	6/8/09	1	6031	Male		On PND6, dam and pups were terminated as moribund.
			2	6032	Male		
			3	6033	Male		
			4	6034	Male		
			5	6035	Female		
			6	6036	Female		
			7	6037	Female		
			8	6038	Female		
2000 mg/L	604	6/9/09					Removed from study due to insufficient litter size - PND4
2000 mg/L	605	6/8/09	1	6051	Male		
			2	6052	Male	53	
			3	6053	Male	58	
			4	6054	Male		
			5	6055	Female	116	
			6	6056	Female	117	
			7	6057	Female	119	
			8	6058	Female		
2000 mg/L	606						Non-Pregnant (0 implantations/0 resorptions)
2000 mg/L	607						Non-Pregnant (0 implantations/0 resorptions)

Litter Data by Individual Dam for the Perinatal Toxicity Dose-Range-Finding Study of XYZ in Harlan Sprague Dawley Rats Administered by Gavage
(This table format may change based on output from the NTP data management system under development)

		SAMPLE														
		PND0[a]										PND4 Litter Standardization				
Concentration	Dam ID	Live Male Pups	Dead Male Pups	Live Female Pups	Dead Female Pups	Litter Observations	Male Litter Weight (g)[b]	Female Litter weight (g)	Total Litter Weight (g)	PND1 Litter Total (Live)	PND4 Litter Total (Live) Pre-standardization	Male Pups on Study	Male Pups Culled	Female Pups on Study	Female Pups Culled	PND4 Litter Total (Live) Post-standardization
0 mg/L	102	9	0	4	0	Normal	55.7	43.9	99.6	13	13	4	5	4	0	8
	103	4	0	7	0	Normal	31.5	51.0	82.5	11	11	4	0	4	3	8
	105	8	0	7	0	Normal	54.4	58.4	112.8	15	15	4	4	4	3	8
	106	4	0	7	0	Normal	28.8	47.6	76.4	11	11	4	0	4	3	8
	107	4	0	2	0	Normal	29.2	14.9	44.1	6	6	0	4	0	2	0
	108	3	0	7	0	Normal	26.5	53.7	80.2	10	10	3	0	5	2	8
Group Total:		29	0	37	0	--	226.1	269.5	495.6	66	66	--	--	--	--	40
Group Mean:		4.8	0.0	6.2	0.0	--	37.7	44.9	82.6	11.0	11.0	--	--	--	--	--
125 mg/L	201	3	0	10	0	Normal	25.5	71.8	97.3	13	13	0	3	0	10	0
	202	6	0	8	0	Normal	46.5	57.2	103.7	14	14	4	2	4	4	8
	203	9	0	6	0	Normal	65.8	43.1	108.9	15	15	4	5	4	2	8
	204	5	0	5	0	Normal	38.8	34.6	73.4	10	10	4	1	4	1	8
	205	5	0	6	0	Normal	38.8	42.6	81.4	11	11	4	1	4	2	8
	206	6	0	9	1	Normal	42.8	60.8	103.6	15	15[c]	4	3	4	4	8
	207	5	0	6	1	Normal	36.7	42.6	79.3	11	11[c]	4	3	4	0	8
	208	7	0	6	0	Normal	44.4	50.3	94.7	13	13	4	3	4	2	8
Group Total:		45	0	57	2	--	339.3	403.0	742.3	102	102	--	--	--	--	56
Group Mean:		5.6	0.0	7.1	0.3	--	42.4	50.4	92.8	12.8	12.8	--	--	--	--	--

a. PND = Postnatal Day.
b. Group weights presented (Male pups weighed together as a group; female pups weighed together as a group)
c. Differing pup gender counts on PND1 and PND4.

QUALITY ASSURANCE STATEMENT
ABC Toxicity Report
Gavage Study of Chemical XYZ In Harlan Sprague Dawley Rats

Study #81-20 (ABC Toxicity Study/Rats)

Listed below are the phases and/or procedures included in the study described in this report which were reviewed by the Quality Assurance Unit, the dates the reviews were performed and findings reported to management. (All findings were reported to the study director or his/her designee at the time of the review.)

Phase/Procedure Reviewed	Review Date	Date of Report To Study Director/Management

Quality Assurance Officer_____ Date _____

Clinical Laboratory Data Form

Laboratory _____ Date Samples Collected _____ Date Assayed _____
Contract Number _____ Instrument Used _____
Test Agent _____ Laboratory Technician _____
(initials and date)
Clinical Laboratory Scientist _____
(signature and date)

Study Information:
Length of Study _____ Current Time-point _____ Route (vehicle) _____
Sample Site _____ Anesthetic _____
Animal Information:
Species _____ Sex _____ Intensity _____

HEMATOLOGY

Animal No.	RBC 10^6/uL	Hct %g/d	Hb fL	MCV pg	MCH g/dL	MCHC 10^3/uL	Retic /100WBC	NRBC 10^3/uL	WBC 10^3/uL	Seg 10^3/uL	Band 10^3/uL	Lymph 10^3/uL	Mono 10^3/uL	Eos 10^3/uL	Baso 10^3/uL	Plat	Comments
1																	
2																	
3																	
4																	
5																	
6																	
7																	
8																	
9																	
10																	

Comments:
1 - Animal died
2 - Insufficient sample
3 - Clotted
4 - Anisocytosis (1-4)
5 - Polychromasia
6 - Howell-Jolly bodies
7 - Heinz bodies
8 - Atypical cells
9 - Smudge/degenerative cells
10 - Toxic change
11 - Poikilocytosis
12 – Other

Clinical Laboratory Data Form

Laboratory _____ Date Samples Collected _____ Date Assayed _____
Contract Number _____ Instrument Used _____
Test Agent _____ Laboratory Technician _____
(initials and date)
Clinical Laboratory Scientist _____
(signature and date)

Study Information:
Length of Study _____ Current Time-point _____ Route (vehicle) _____
Sample Site _____ Anesthetic _____
Animal Information:
Species _____ Sex _____ Intensity _____

CLINICAL CHEMISTRY

Animal No.	ALT U/L	ALP U/L	SDH U/L	Bile Acids umol/L	CK U/L	TP g/dL	Alb g/dL	BUN mg/dL	Creatinine mg/dL	Glucose mg/dL	Comments
1											
2											
3											
4											
5											
6											
7											
8											
9											
10											

Comments: 1 - Animal died 3 - Hemolysis (1-4) 5 - Icterus (1-4) 7 - Other _____
2 - Insufficient sample 4 - Lipemia (1-4) 6 - Dilution _____

XIII. APPENDICES

APPENDIX 1 - Dose Analysis and Method Performance Evaluation

Contents

Appendix 1. PRESTART CHEMISTRY REPORT

Dose Analysis and Method Performance Evaluation:

Methods developed by the Testing Laboratory for dose analysis shall be described. A prestart method performance evaluation shall be conducted according to the following general protocol and included in the report.

1. Rationale:

   The purpose of the protocol is to provide a uniform procedure for investigators to confirm satisfactory performance of analytical chemistry methods specified by the NTP prior to initiating their studies. These tests shall be conducted over the dose concentration range used for the toxicity tests.

2. Key Elements:

   The key elements for the performance evaluation include the following:

   a. An indication of the precision of the method at specified concentrations.

   b. Confirmation by statistical and visual inspection that the response versus concentration is linear over the specified concentration range.

   c. An indication of blank vehicle contribution to responses seen in spiked vehicle determinations.

   d. Estimation of recovery (percent) of the chemical from spiked vehicle at specified concentrations.

   e. Determination of the percent relative error.

   f. Estimates of the measurement limits.

   To aid in the execution of the statistical requirements, all the computational formulae will be given in the text.

3. Spiked Vehicle Standard Preparation

   Prepare spiked vehicle standards at six different concentrations using two independently prepared stock standards of different concentrations (Stock A and Stock B). Make spiked vehicle standards in triplicate at each concentration. Prepare a vehicle blank in triplicate. The concentrations of the spiked standards should be arranged so that each standard comes from alternate stock solutions (Table 1). Measure the response from a single analysis of each vehicle standard and blank.

Table 1:

Concentration of Vehicle Blank	Concentrations of Vehicle Standards					
X_{O_1}	X_{B_4}	X_{A_7}	$X_{B_{10}}$	$X_{A_{13}}$	$X_{B_{16}}$	$X_{A_{19}}$
X_{O_2}	X_{B_5}	X_{A_8}	$X_{B_{11}}$	$X_{A_{14}}$	$X_{B_{17}}$	$X_{A_{20}}$
X_{O_3}	X_{B_6}	X_{A_9}	$X_{B_{12}}$	$X_{A_{15}}$	$X_{B_{18}}$	$X_{A_{21}}$

Where the subscripts A and B denote the stock standards, A and B, which were used to prepare the vehicle standards and O signifies the blank.

Compute the mean response, Y_{f_i}, for each concentration, X_{f_i}, of the standards and the blanks (Table 2). Compute the standard deviation, S_i, for each concentration (equation 1).

Table 2:

Response of Vehicle Blank	Responses of Vehicle Standards					
Y_{O_1}	Y_{B_4}	Y_{A_7}	$Y_{B_{10}}$	$Y_{A_{13}}$	$Y_{B_{16}}$	$Y_{A_{19}}$
Y_{O_2}	Y_{B_5}	Y_{A_8}	$Y_{B_{11}}$	$Y_{A_{14}}$	$Y_{B_{17}}$	$Y_{A_{20}}$
Y_{O_3}	Y_{B_6}	Y_{A_9}	$Y_{B_{12}}$	$Y_{A_{15}}$	$Y_{B_{18}}$	$Y_{A_{21}}$

$\overline{Y}_{O_j} \pm S_j$ $\overline{Y}_{B_j} \pm S_j$ $YA_j \pm S_j$ $\overline{Y}_{B_j} \pm S_j$ $\overline{Y}_{A_j} \pm S_j$ $\overline{Y}_{B_j} \pm S_j$ $\overline{Y}_{A_j} \pm S_j$

Where j represents a set of three standards at one concentration (j=0–6).

So, the standard deviation, S_j for each triplicate standard set is given by:

$$S_j = \left\{ \frac{1}{(n-1)} \left[\sum_{k=a}^{b} Y_{f_k}^2 - \frac{1}{n} \left(\sum_{k=a}^{b} Y_{f_k} \right)^2 \right] \right\}^{1/2} \qquad [1]$$

where a and b are 1 and 3 (for the blanks), or 4 and 6 (for the first set of triplicate standards), or 7 and 9, 10 and 12, 13 and 15, 16 and 18, or 19 and 21 for each of the other sets of triplicate standards.

4. Solvent Standard Preparation

Prepare a series of solvent standard solutions with the same final concentrations (i.e. after any extraction, dilution, or concentration step) as were used for measurements on the vehicle standards (Table 3). Use the same standard stock solutions (A and B), prepared in E. I. B.1., for making these solutions. Prepare a reagent blank in the same solvent as the standards. Measure the responses on the same analytical system used for the vehicle standards (Table 4).

Table 3

Reagent Blank Concentration	Solvent Standard Concentrations					
X_{SO}	X_{S1}	X_{S2}	X_{S3}	X_{S4}	X_{S5}	X_{S6}

Table 4

Reagent Blank Responses	Solvent Standard Responses					
Y_{SO}	Y_{S1}	Y_{S2}	Y_{S3}	Y_{S4}	Y_{S5}	Y_{S6}

5. Calculations

a. Calculate the linear regression equations for the vehicle and solvent standard curves. Do not correct the response for experimental blank values. For spectrophotometric determinations, zero the instrument using the solvent only, then compare the blank value with the Y intercept (b0) obtained from the linear regression equation. Linear regression parameters, the slope (b1), and the intercept (b0), are calculated as follows (equations 2 and 3):

$$b_1 = \frac{\sum_{k=1}^{21} X_{f_k} Y_{f_k} - \frac{\left(\sum_{k=1}^{21} X_{f_k}\right)\left(\sum_{k=1}^{21} Y_{f_k}\right)}{n}}{\sum_{k=1}^{21} X_{f_k}^2 - \frac{\left(\sum_{k=1}^{21} X_{f_k}\right)^2}{n}} \qquad [2]$$

$$b_0 = \frac{1}{n}\left(\sum_{k=1}^{21} Y_{f_k} - b_1\left(\sum_{k=1}^{21} X_{f_k}\right)\right) \qquad [3]$$

Where:

X_{f_k} = Prepared concentrations of the standards

Y_{f_k} = Values of the response corresponding to X_{f_k}

b. Calculate the correlation coefficient, r, for the solvent and vehicle standard curve data as follows (equation 4):

$$r = \frac{\sum_{k=1}^{21} X_{f_k} Y_{f_k} - \frac{\sum_{k=1}^{21} X_{f_k} \sum_{k=1}^{21} Y_{f_k}}{n}}{\left[\left(\sum_{k=1}^{21} X_{f_k}^2 - \frac{\left(\sum_{k=1}^{21} X_{f_k}\right)^2}{n}\right)\left(\sum_{k=1}^{21} Y_{f_k}^2 - \frac{\left(\sum_{k=1}^{21} Y_{f_k}\right)^2}{n}\right)\right]^{\frac{1}{2}}} \qquad [4]$$

For clarification of the variables used in the above equations, see Figure 1 below:

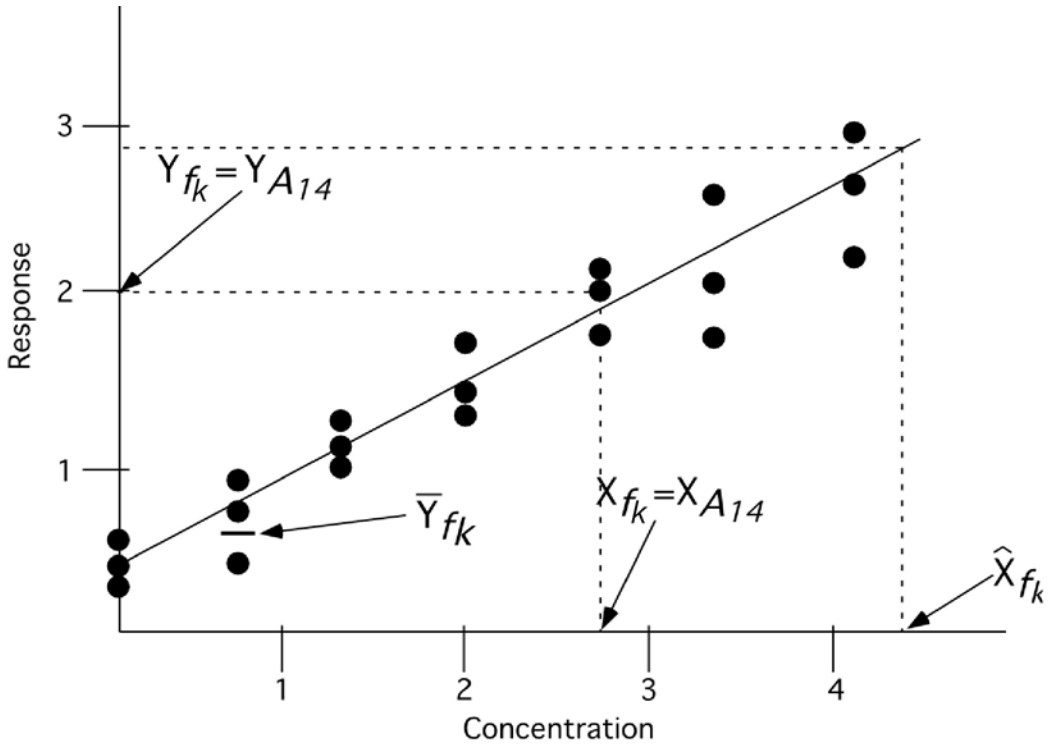


Figure 1

c. To estimate precision, calculate the percent relative standard deviation (% RSD) for each vehicle standard analyzed in triplicate (equation 5).

$$\%RSD = \left[\frac{\left[\sum_{j=1}^{3} Y_{f_j} - n\overline{Y_{f_j}} \right]^{\frac{1}{2}}}{\frac{n-1}{\overline{Y_{f_j}}}} \right] \times 100 \qquad [5]$$

Where $\overline{Y}_{f_i}$ is the average response for each i[th] set of vehicle standards at a particular concentration or blank; I represents each replicate standard.

d. Determine the relative error of each found (calculated) concentration compared to each prepared concentration as follows:

i.) Calculate the concentration from the measured responses for the vehicle standards using the regression line (equation 6). Use the mean responses for the vehicle standards and the blanks to calculate the mean found concentration.

$$\hat{X}_{f_k} = \frac{Y_{f_k} - b_0}{b_1} \qquad [6]$$

where $\hat{X}_{f_k}$ s the found concentration of the standards, and Y_{f_k} is the instrument response for each standard (or the mean response for triplicates).

ii) Calculate the relative error as follows (equation 7):

$$\text{RelativeError} = \frac{\hat{X}_{f_k} - X_{f_i}}{X_{f_i}} \times 100 \qquad [7]$$

where X_{f_i} is the prepared concentration of each vehicle standard.

f. Determine the measurement limits as defined below:

The limit of detection (LOD) is defined as three times the standard deviation of the blank (if there is no blank value the standard deviation of the lowest standard concentration is to be used).

The limit of quantitation (LOQ) is defined as ten times the standard deviation of the blank (if there is no blank value the standard deviation of the lowest standard concentration is to be used).

The experimental limit of quantitation (ELOQ) is defined as the lowest vehicle standard concentration that has been analyzed that has a relative error of within 10% of target and a relative standard deviation of $\leq$ 10%.

BLANK PAGE

APPENDIX 2 - Protocols for the Analysis of Dosing Vehicles Used in NTP Toxicity Studies

I. Analysis of Corn Oil for Peroxide

A. Rationale:

Any batch of corn oil used in the testing program shall be analyzed for peroxides before it is first used and at bimonthly intervals thereafter while it is in use. Corn oil must be purchased in batches no smaller than two gallons (7 Kg) per chemical for which it is the dose vehicle, unless substantially less than this quantity is being used monthly. Purchase of lesser quantities needs to be approved by the Project Officer. All corn oil must be stored at $5^{\circ}C \pm 3^{o}$.

The following is a standard analytical procedure that should be used for these analyses. The method employed is the Official Method of the A.O.C.S. (1972). (Alternate methods need to be approved by the NTP Project Officer.) It determines all substances, in terms of milli-equivalents (meq) of peroxide per thousand grams of sample, which oxidize aqueous iodide under the conditions of the test. These are generally assumed to be peroxides or other similar products of fat oxidation. The method is highly empirical, and any variation in procedures may affect the results.

B. Method:

A 5.0 gram sample of the corn oil to be analyzed is quantitatively transferred into a 250 ml titration flask and 30 ml of glacial acetic acid:chloroform (60:40 v/v) is added. The mixture is stirred until the corn oil has completely dissolved. One-half ml of a saturated aqueous potassium iodide solution is added. The test solution is stirred thoroughly and allowed to stand for exactly one minute, after which 30 ml of distilled water is added. The iodine liberated by the peroxides in this solution is titrated potentiometrically with standard 0.005 N sodium thiosulfate solution, stirring vigorously to ensure thorough mixing. An automatic titrator with platinum disk working electrode and silver - silver chloride reference electrode is convenient. Titrations shall be run in triplicate. A blank titration of reagents will be run on the day the oil sample is analyzed; the blank will not titrate more than 1.0 ml of the 0.005 N sodium thiosulfate standard solution. Peroxide number, expressed in milli-equivalents of peroxide per kilogram of oil (meg/Kg), is calculated as follows:

$$\text{Peroxide Number} = \frac{(V-B) \times N \times 1000}{W}$$

V = volume (in ml) of thiosulfate solution required for titration of oil sample

B = volume (in ml) of thiosulfate solution required for titration of reagent blank

N = normality of sodium thiosulfate solution

W = weight of oil sample in grams

C. Additional Requirements:

The Testing Laboratory shall develop an analytical procedure (or SOP) based on this methodology. When reporting results, the SOP can be cited by reference. When reporting follow-up analyses, the dates and results of all previous analyses of the batch of corn oil shall be included for comparison. These data shall be summarized in the first Monthly Progress Report after the analysis. The report shall indicate which test chemical or chemicals the peroxide analysis is for and a copy of the analysis shall be included in the raw data submitted to the Archives for each chemical (microfiche copies are acceptable.)

D. Limits:

Corn oil with a peroxide number equal to or greater than 3 meq/Kg shall be considered rancid for purposes of this program and must be replaced immediately.

E. Characteristics and approximate composition of corn oil (Table 1):

Table 1

Calories per gram		8.9
Iodine value		127
Peroxide value (meq/L)		1.6
Anisidine value		2.3
Saponification value		191
Color		2.3 R/14 Y
Components		
Glycerides*		>98.7 %
Unsaponifiable matter*		1.25 %
Free fatty acids		0.04 %
Phosphorus		0.5 ppm
Sodium		0.1 ppm
Calcium		0.1 ppm
Magnesium		<0.1 ppm
Organo-chloride pesticide residues		<10 ppb†
Aflatoxin		<0.5 ppb†
Heavy Metals (Pb, Cu, Ni, Fe)		<0.1 ppm†
Estrogenic activity	Not detected	<5 ppb†
Fatty Acid, grams/100 grams of corn oil		
Total fatty acids		94.3
C12:0		trace
C14:0		trace
C16:0		9.5
C16:1		0.2
C18:0		2.3
C18:1		25.4
C18:2		55.1
C18:3		1.0
All others		0.8
Essential fatty acid (lipoxydase)		56.8
Unsaponifiables *, % of oil		1.25
Phytosterols		>1.0
Stigmasterol		0.07
Beta-Sitosterol		0.8
Gamma-Sitosterol or Campesterol*		0.2
Tocopherols - total		0.098
Alpha-Tocopherol		0.014
Gamma-Tocopherol		0.084
Delta-Tocopherol		<0.001
Ubiquinone (coenzyme Q-9)*		0.02
Squalene*		trace
Carotenoids*		trace

*From past historical experience; not analyzed
†Limits of detection

II. Protocols for the Analysis of Ethanol used in Toxicity Studies

A. Identity by Infrared Spectrophotometry

1. Prepare a thin film of the sample by placing 1 to 2 drops between silver chloride plates. Be sure no air bubbles are trapped in the cell.

2. Obtain an IR spectrum of the sample from 600 to 4000 cm^{-1} using a suitable infrared spectrophotometer. Adjust the instrument settings or cell thickness to obtain a baseline of approximately 80% transmission, keeping the largest absorbances greater than or equal to 10% transmission.

B. Purity Assessment by Chromatography

1. Sample Preparation

Prepare and analyze two test article (ethanol) solutions. Prepare solution A by volumetrically pipetting 0.5 mL of test article and 0.5 mL of cyclohexanone (internal standard) into a 100-mL volumetric flask. Dilute the contents of the flask to volume with water and mix by inversion. Prepare solution B by delivering a 0.5-mL portion of cyclohexanone into a 100-mL volumetric flask, diluting the contents of the flask to volume with test article and mixing by inversion. Prepare an internal standard blank solution by pipetting 0.5 mL of cyclohexanone into a 100-mL volumetric flask. Bring the contents of the flask to volume with water and mix by inversion.

2. Analysis

Analyze the two solutions (A and B), the internal standard blank, a portion of the neat test article, and water blank. Use the instrument system and parameters described below.

3. System Suitability

Evaluate the analytical system described above for precision, theoretical plates, resolution, and tailing factor, according to USP guidelines. Calculate precision using the average response ratio for the ethanol peak obtained from six injections of solution A. Evaluate the tailing factor at 5% base height for the ethanol peak of a single injection of solution A. Evaluate resolution for the ethanol and cyclohexanone peaks of a single injection of solution A. Calculate theoretical plates for the ethanol peak of a single injection of solution A.

4. Instrument System and Parameters

Instrument:	Varian 3700 with Varian 8000 autosampler (or equivalent)
Column:	DB-Wax, 30 m x 0.53 mm ID, 1-mm film thickness, fused silica
Temperature Program:	40^{o} C (5-min hold) to 220°C (5-min hold) at 10°C/min
Injection Volume:	1 μL
Mode:	Direct
Detector:	Flame ionization
Attenuation:	32 x 10^{-11}
Temperatures:	
Inlet:	150°C

Detector:	220°C
Carrier Gas:	Helium
Flow Rate:	10 mL/min
Makeup Gas:	Nitrogen
Flow rate:	20 mL/min
Air Flow Rate:	300 mL/min
Hydrogen Flow Rate:	30 mL/min
Retention Times:	
Ethanol:	2.3 minutes
Internal standard:	9.9 minutes

Set the attenuation so that a 60% to 80% pen deflection is obtained for the internal standard peak (approximately 32 x 10^{-11} AFS). Use the chromatograms from injection of the neat ethanol sample and solution A to correlate observed peaks with their respective retention times. Also use the chromatograms from the neat ethanol sample and the water solvent to determine that there are no interferences on the internal standard peak.

5. Calculations;

Calculate the relative response factor (RRF_A) for the ethanol peak observed from solution A using the following formula:

$$RRF_A = \frac{\text{Peak area of ethanol x 200}}{\text{Peak area of internal standard}}$$

Calculate the relative response factors for each impurity observed from injection of solution B using the following formula:

$$RRF_i = \frac{\text{Peak area of impurity}}{\text{Peak area of internal standard}}$$

Calculate the relative concentration of each impurity in the sample of ethanol using the following formula:

$$\text{Relative concentration (\%)} = \frac{RRF_i \times 100}{RRF_A}$$

Report the retention times and relative concentrations for any impurities with relative concentrations greater than or equal to 0.1%.

6. Limits

Ethanol IR spectrum must match with a library reference spectrum. Any detectable benzene is not acceptable for NTP studies. Material less than 99.9% is not acceptable.

7. Benzene Content Screening Analysis

If ethanol is obtained from a source other than the one recommended, the vehicle shall be screened for benzene content using the following method. A sample of the neat test article should be analyzed along with a series of benzene standards, prepared at concentrations from approximately 1 to 10 ppm. This would only be necessary on the initial receipt of each lot of vehicle. The DB-5 column, produced by J.& W. Scientific, is available from the same distributors as the DB-Wax column.

Instrument:	Varian 3700 with Varian 8000 autosampler (or equivalent)
Column:	DB-5, 30 m x 0.53 mm ID, 1.5-mm film thickness, fused silica
Temperature Program:	40°C (3-min hold) to 200°C (3-min hold) at 10°C/min
Injection Volume:	1 mL
Mode:	Direct
Detector:	Flame ionization
Attenuation:	32×10^{-11}
Temperatures:	
Inlet:	150°C
Detector:	220°C
Carrier Gas:	Helium
Flow Rate:	10 mL/min
Makeup Gas:	Nitrogen
Flow rate:	20 mL/min
Air Flow Rate:	~ 300 mL/min
Hydrogen Flow Rate:	~ 30 mL/min

III. Protocols for the Analysis of Acetone

A. Identity by Infrared Spectrophotometry

1. Place five to six drops of acetone into an infrared gas cell[c.] equipped with sodium chloride windows and allow the sample to volatilize (volatilization may be accomplished by slight warming of the cell with the hands).

Obtain a spectrum for the sample from 600 to 4000 cm^{-1} using a suitable infrared spectrophotometer. Adjust the instrument settings or sample concentration to obtain baselines of about 80% transmission, keeping the largest absorbances at greater than or equal to 10% transmission.

B. Purity Assessment by Chromatography

1. Sample Preparation

Prepare and analyze two test article (acetone) solutions. Prepare solution A by volumetrically pipetting 0.5 mL of test article and 0.5 mL of cyclohexanone (internal standard) into a 100-mL volumetric flask. Dilute the contents of the flask to volume with water and mix by inversion. Prepare solution B by delivering a 0.5 mL portion of cyclohexanone into a 100-mL volumetric flask, diluting the contents of the flask to volume with test article and mixing by inversion. Prepare an internal standard blank solution by pipetting 0.5 mL of cyclohexanone into a 100-mL volumetric flask. Bring the contents of the flask to volume with water and mix by inversion.

2. Analysis

Analyze the two solutions (A and B), the internal standard blank, a portion of the neat test article, and a water blank. Use the instrument system and parameters described below.

3. System Suitability

Evaluate the analytical system described above for precision, theoretical plates, resolution, and Tailing factor, according to U.S.P. guidelines. Calculate precision using the average response ratio for the acetone peak obtained from six injections of solution A. Evaluate the tailing factor at 5% base height for the acetone peak of a single injection of solution A. Evaluate resolution for the acetone and cyclohexanone peaks of a single injection of solution A. Calculate theoretical plates for the acetone peak of a single injection of solution A. A comparison between the criteria for the current method and those proposed for the modified method

4. Instrument System and Parameters

Instrument:	Varian 3700 with Varian 8000 autosampler (or equivalent)
Column:	DB-Wax, 30 m x 0.53 mm ID, 1-mm film thickness, fused silica
Temperature Program:	40°C (5-min hold) to 220°C; (5-min hold) at 10°C/min
Injection Volume:	1 mL
Mode:	Direct
Detector:	FID
Attenuation:	32×10^{-11}

Temperatures:
- Inlet: 150°C
- Detector: 220°C

Carrier Gas: Helium
- Flow Rate: ~ 10 mL/min

Makeup Gas: Nitrogen
- Flow Rate: ~ 20 mL/min

Air Flow Rate: ~ 300 mL/min

Hydrogen Flow Rate: ~ 30 mL/min

Retention Times:
- Acetone: ~ 1.2 min
- Cyclohexanone (internal standard): ~ 9.9 min

Set the attenuation so that a 60% to 80% pen deflection is obtained for the internal standard peak (approximately 32 x 10^{-10} AFS). Use the chromatograms from injection of the neat acetone sample and solution A to correlate observed peaks with their respective retention times. Also use the chromatograms from the neat acetone sample and the water solvent to determine that there are no interferences on the internal standard peak.

5. Calculations

Calculate the relative response factor (RRF_A) for the acetone peak observed from solution A using the following formula:

$$RRF_A = \frac{\text{Peak area of acetone x 200}}{\text{Peak area of internal standard}}$$

Calculate the relative response factors for each impurity observed from injection of solution B using the following formula:

$$RRF_i = \frac{\text{Peak area of impurity}}{\text{Peak area of internal standard}}$$

Calculate the relative concentration of each impurity in the sample of ethanol using the following formula:

$$\text{Relative concentration (\%)} = \frac{RRF_i \times 100}{RRF_A}$$

Report the retention times and relative concentrations for any impurities with relative concentrations greater than or equal to 0.1%.

6. Limits

IR spectrum must match with a library reference spectrum. Material less than 99.9% pure is not acceptable.

IV. Protocols for the Analysis of Methylcellulose (Dosing Vehicle) used in Toxicity Studies

A. Receipt of Bulk Chemical

When the bulk chemical is received, remove 0.5 g portions for each subsequent analysis. Place each sample in an appropriately labeled glass vial equipped with a Teflon®-lined screw cap, then tightly close and seal the vial, and store at –20° C. Use this material in subsequent analyses, at intervals specified by the NTP, as the reference standard. Store the remainder of the bulk material at room temperature (~ 25° C).

B. Identity by Infrared Spectrophotometry

1. Prepare separate potassium bromide discs containing approximately 3% methylcellulose for both the bulk chemical and the reference standard.

2. Obtain a spectrum for the two samples from 600 to 4000 cm^{-1} using a suitable infrared spectrophotometer. Adjust the instrument settings or sample concentration to obtain baselines of about 80% transmission, keeping the largest absorbances at greater than or equal to 10% transmission.

C. Purity Analysis by Methoxy Group Determination

1. Procedure

This analysis (duplicate samples) may be performed by the Contractor or by an independent laboratory. If an independent laboratory does the analysis, it is the responsibility of the Contractor to verify the quality assurance compliance of that laboratory.

2. Calculations

a. Calculate the average determined values (%) for methoxy group content of the bulk chemical and the reference standard to the tenths place.

b. Calculate the relative purity (%) for the bulk chemical to the tenths place by dividing the average determined value (%) for the bulk chemical by the average determined value for the reference standard and multiplying by 100.

3. Limits

IR spectrum must match with a library reference spectrum. Results of analysis for methoxy group content must be 27.5 to 31.5 %.

APPENDIX 3 - Laboratory Animal Management Appendix

Contents of Appendix

HEAT TREATED HARDWOOD BEDDING
MAXIMUM LEVELS OF CONTAMINANTS

CHEMICAL CONTAMINANTS

Pesticide Residues (ppm)	
Chlorinated Hydrocarbons	
Alpha BHC	< 0.02
Beta BHC	< 0.02
Lindane	< 0.02
Aldrin	< 0.02
Heptachlor Epoxide	< 0.02
Dieldrin	< 0.02
Endrin	< 0.02
DDT	< 0.03
DDD	< 0.02
DDE	< 0.02
Organophosphates	
Diazinon	< 0.10
Ethyl Parathion	< 0.03
Methyl Parathion	< 0.03
Malathion	< 0.05
Ethion	< 0.02
Ronnel	< 0.03
Triothion	< 0.03
Polychlorinated Biphenyls	< 0.20
Pentachlorophenol	< 0.10
Aflatoxins (ppm)	< 0.01
Heavy Metals (ppm)	
Lead	< 0.50
Mercury	< 0.10
Cadmium	< 0.10
Arsenic	< 0.20

MICROBIOLOGICAL CONTAMINANTS *

Standard Plate Count	< 100
Coliform	< 10
Pseudomonads	Neg
Yeast and Molds	< 10
Salmonella/Shigella	Neg

* All values in total organisms/gm of bedding

NIH-07 or NTP-2000 DIET - LIMITS OF CONTAMINANT LEVELS

AFLATOXINS
(Maximum PPB)

Total	5
B1	2

NITROSAMINES
(Maximum PPB)

Total	15
(Volatile)	
N-nitroso Dimethylamine	10

HEAVY METALS
(Maximum PPM)

Lead	1.00
Cadmium	0.15
Mercury	0.05
Arsenic	0.50
Selenium	0.50

CHLORINATED HYDROCARBONS
(Maximum PPM)

BHC	
Alpha	0.02
Beta	0.02
Delta	0.02
Lindane	0.02
Heptachlor	0.02
DDE	0.02
DDD	0.02
DDT	0.03
HCB	0.08
Mirex	0.02
Methoxychlor	0.05
Dieldrin	0.02
Endrin	0.02
Telodrin	0.02
Chlordane	0.05
Toxaphene	0.10

ORGANOPHOSPHATES
(Maximum PPM)

Chloropyrifos-methyl	0.10
Ronnel	0.03
Ethion	0.02
Trithion	0.05
Diazinon	0.20
Methylparathion	0.03
Ethylparathion	0.03
Malathion	0.50
Endosulfan I	0.02
Endosulfan II	0.02
Endosulfan Sulfate	0.03

PCB'S
(Maximum PPM) 0.20

MISCELLANEOUS
(Maximum Limits)

Nitrate (N PPM)	20
Nitrite (N PPM)	5
BHA (PPM)	10
BHT (PPM)	5

Total Bacterial Plate Count

(CFU/Gm)	1000
Coliform (MPN/Gm)	10
E. Coli (MPN/Gm)	10
Salmonella (/Gm)	Neg

RATING OF THE FEED FOR CONTAMINANTS

Maximum Points - 100
95 to 100 - use the feed
91 to 94 - May use it but replace with a new batch within four weeks
90 and below - reject the feed

1. If all contaminants are at less than the specifications, rating for that batch of feed will be 100.

2. Aflatoxin - Deduct 1 point for each PPB above the specification. If the aflatoxin level is more than 10 PPB, the feed shall not be used.

3. Nitrosamines - Deduct 1 point for each 2 PPB above the specifications.

4. Heavy Metals:

   Lead, Arsenic, and Selenium - Deduct 1 point for each 0.2 PPM above the specifications.

   Cadmium and Mercury - Deduct 1 point for each 0.02 PPM above the specifications.

5. PCB's - Deduct 1 point for each 0.02 PPM above the limit.

   Pesticides - Deduct 1 point for each increase equivalent to the maximum allowable level.

6. Miscellaneous contaminants - Deduct 1 point for each increase equivalent to the maximum allowable level. If microbiological contaminants are 2X the limits, a new sample is to be tested for possible contamination during sampling. If the repeat sample confirms the original results and the total bacterial count is > 5000 cfu/gm, the product was not irradiated properly and cannot be accepted as an irradiated product.

WATER ANALYSIS

Laboratories must demonstrate that water provided for animal use meets US/EPA National Primary Drinking Water Regulations. In addition, the components and contaminants listed below are to be determined and assessed.

Metals (mg/L)

Na	Ba
K	Sr
Ca	B
Mg	P
Al	Cr
Fe	Cu
Mn	Zn

Chlorinated Hydrocarbons (mg/L)

Aldrin
Dieldrin
DDT Related Substances

Organophosphates: (mg/L)

Phorate
Diazinon
Methyl Parathion
Malathion
Parathion
Endosulfan
Carbophenothion

HUMANE ENDPOINTS IN TOXICOLOGY STUDIES USING RODENTS

The final decision for euthanasia of moribund animals shall be made by the Laboratory Veterinarian or an experienced scientist, and shall not be left to the discretion of the technicians. Major reasons for euthanasia of animals during the course of a study include: a) large masses and other conditions preventing eating and drinking, b) major injuries and lesions such as non-healing ulcers related to husbandry and treatment, c) diseases and conditions indicating severe pain, and d) adequate indication that the animal may not survive until the next observation. Other conditions for euthanasia of rodents in long-term studies are listed below. These criteria should be supplemented with professional judgment for euthanasia of animals during the course of a study.

- Loss of 20 to 25% body weight in less than one week
- Gradual but sustained decline in body weight indicating partial and sustained anorexia
- Prolonged unhealthy appearance such as rough coat, hunched posture and distended abdomen
- Prolonged diarrhea leading to emaciation
- Prolonged or intense diuresis leading to emaciation
- Persistent coughing, wheezing and respiratory distress
- Paralysis and other nervous disorders leading to anorexia and continuous decline in body weight
- Bleeding from natural orifices not due to minor injuries
- Persistent self-induced trauma complicating minor injuries
- Microbial infections interfering with toxic and carcinogenic responses

Adapted from Rao, G.N. and Huff, J (1990), Refinement of long-term toxicity and carcinogenesis studies, Fundamental and Applied Toxicology 15: 33-43.

APPENDIX 4 - Individual Animal Necropsy Record

I. INTRODUCTION

There shall be a necropsy record for each animal on study that is necropsied. NTP is currently in the process of implementing a data collection and management system for reproductive and developmental toxicity studies that shall include collection of necropsy and trimming data; this section lists the types of data that must be collected for an Individual Animal Necropsy Record (IANR). The IANR Form is the paper or electronic form used to record the gross pathology data during necropsy and trimming for each animal on the study. The NTP Pathology Code Table (PCT) contains the vocabulary used for the completion of the IANR. A sample form filled in with the type of data to be collected, as well as blank forms, are included below.

This document will be amended for the NTP Provantis data collection system prior to that system being required for data collection and management.

II. CORRECTION PROCEDURES

During the course of completing the paper IANR form, incorrect entries may be made. These errors may be discovered by the pathologist during his review of the form prior to signing the Pathologist Signature Field (26), or during a quality control check or audit of the data forms. To correct an entry, a single line is drawn through the entry so as not to obscure the original entry. The correct entry is made and a footnote callout (a circled number is entered near the incorrect data). The reason for the change, the initials of the person making the change, and the date, preceded by the corresponding circled callout number, are entered in Field 30, called “Correction Comments”.

III. DESCRIPTION OF FIELD

Each field on the form has a field number and/or name. Listed below are the fields on the form. The "Description" heading describes the appropriate entries for the fields. Guidelines and comments concerning the requirements for the completion of the applicable fields are provided below.

Note: The forms are designed to be <u>species/sex</u> specific. It is important that the appropriate form is used for each animal.

FIELD NO:	NAME:	DESCRIPTION
1.	FACILITY:	Enter the correct two-character NTP acronym for the test facility.
2.	TEST ARTICLE:	Enter the full name of the test article used on this study. DO NOT use an acronym or generic name to designate the test article. In addition, if diet restricted, stop study or other special case is involved, note this beside the test article name, i.e. Test Article XYZ (Stop Study).
3.	PHASE:	Indicate the phase of the study by marking (X) in the appropriate box.
4.	DAYS ON TEST:	Enter the number of days the animal was on test inclusive of the day of death.
5.	DOSE:	Enter the dose for the animal (i.e., mg/kg, ppm). If the animal is an untreated control or vehicle control, enter accordingly.
6.	ROUTE:	Enter the route of administration, using the appropriate acronym listed below. Route may have multiple entries. Separate entries with a hyphen. DF = Dosed Feed GV = Gavage DW = Dosed Water SP = Dermal
7.	STUDY-TEST NO:	Enter the TDMS or NTP study-test number.
8.	SPECIES/STRAIN:	The IANR is specific for animal species/strain/sex and these fields have already been completed. However if a species/strain other than the Harlan Sprague Dawley rat and CD-1 mouse are used, the correct species/strain is to be placed in this field.
9.	SEX:	The IANR is specific for species/strain/sex, and these fields have already been completed.
10.	ANIMAL NO:	Enter the unique animal number. This entry is right justified. Fill in any empty boxes with a dash or a zero.
11.	HISTO NO:	Enter the unique histology number assigned to the animal. This entry is left justified. The same number may be used for the histology number and the animal number.
12.	DEATH DATE:	Enter the date the animal was found dead, sacrificed moribund, or a scheduled sacrifice. Enter in the format MMDDYY.
13.	BODY WEIGHT: (G)	Enter the weight of the animal to the nearest tenth of a gram at the time of necropsy. This entry is right justified.
14.	DISPOSITION:	Mark (X) the appropriate box to indicate the disposition of the animal. The long text of valid dispositions are as follows: TSAC = Terminal Sacrifice NATD = Natural Death MSAC = Moribund Sacrifice ACCK = Accidentally Killed SSAC = Scheduled Sacrifice (Interim) DACC = Dosing Accident OTHER*

COMMENT: The disposition of OTHER is to be used to designate missing, mis-sexed, etc. The use of this disposition requires the entry of an explanation in the DEFINE OTHER blank. Animals removed as “OTHER” are to be discarded without necropsy. The disposition of missing or mis-sexed requires that the header of the form be completed and submitted with the study. An animal that is humanely killed for a reason unrelated to the chemical, such as broken back, or fractured skull, is to be designated as ACCK. This is not to be confused with a MSAC animal.

15. CONDITION: Mark (X) the appropriate box to indicate the condition of the animal at the time of receipt for necropsy. The long text of the valid conditions is as follows:

FRESH
AUTO/TT = Autolysis/Tissue Taken
PT CANN = Partially Cannibalized
CANN/NTT = Cannibalized/No Tissue Taken
OTHER*

*DEFINE OTHER: Enter free text in this blank to explain the use of OTHER. If there is not sufficient room, use Field 24, Notes.

COMMENT: All tissues must be taken at necropsy unless extreme conditions exist. Therefore, the use of CANN/NTT is not to be used without the permission of the assigned pathologist. Autolysis shall not preclude saving of the tissues in formalin.

16a. ID INFORMATION: Mark (X) in the "verified" box after confirming the identification of the animal,
OR
1) If the animal's identification is partially or completely unreadable, explain the condition of the identifier under comment and indicate the animal number as read, using dashes for numbers that are unreadable. If the animal number cannot be read at necropsy, then the animal is to be uniquely re-identified and this number entered in the ID number field
2) If the tattoo is different from the animal number in box 10, explain under comment.

16b. WET TISSUE REVIEW: At the time of wet tissue review, check appropriate box – PERFORMED OR NOT REQUIRED - for this animal. Initial and date entry.

17a. ORGAN List of organs to be taken at necropsy. The tissue list required for specific studies is dependent on the study protocol. Blank spaces are provided to write in organs that are not listed.

The following is a discussion of the procedure to be used for specific accountable sites and organs.

Organs - Lesions that occur in large blood vessels are described in Fields 18 through 22, but lesions of small vessels are described within the organs containing the vessel. The prosector and the pathologist are to limit the use of the organ blood vessels to the sites listed in the PCT Table.

"Tissue NOS" (no organ specified) is an available term used to cover situations in which the site of a mass is not clearly determined. The identification of the Tissue NOS is determined histopathologically. A mass in the inguinal area could be a mammary gland neoplasm, a preputial gland abscess, or lymphadenopathy. If the term Tissue NOS is used, then any involved

organs are to be designated as "A"; a free text description of the area in which the mass was observed is to be written in Field 24, Notes. The microscopic evaluation will key the pathologist to the correct organ for the observation. (See 24. Notes: below.)

Gross description of blood pertains to abnormal color or consistency. The prosector is responsible for observing the gross appearance of blood at the time of necropsy for all animals.

Site Accountability - The accountable sites of an organ are indented directly under the appropriate organ. If lymph nodes other than those listed on the form are observed to be abnormal, then the blank designated "Other" under Lymph Node is to be marked "B" and the site designated in Field 19.

17b. NEC: Enter one of the letters listed below to indicate the condition of the organ at the time of necropsy.

A = Normal
B = Observed. If this entry is used, an entry is required in fields 18-22.
C = Missing. If one of a paired organ is missing, enter this fact in the notes section, (i.e., right adrenal missing). Record the appropriate status for the contra-lateral organ in Field 17b.
D = Present, not examined. This term is used for organs which are normally difficult to evaluate grossly due to size or location, (i.e., spinal cord).

17c. WGT (mg): Enter the weight of the organ in milligrams. If an automated data system is used to collect the weight then this field does not have to be completed on the paper form (if used). The form must, however, indicate that the data has been recorded elsewhere. If the protocol does not require organ weights, the column is to be left blank.

Fields 18 - 24 represent the prosector's, trimming technician's or pathologist's observations of gross abnormalities at the time of necropsy or trimming. All observations receive a sequential Trace Gross Lesion (TGL) number as defined in Field 23 and require an accountability action at the time the pathologist performs the microscopic evaluation of the animal. For liver and lung, up to five nodules/masses shall be recorded individually (the largest). When there are more than five, a note is to be included in section 24 indicating that there were “greater than five” for that organ.

18a. TRIM ID: If an observation is made by the trimming technician, the trimmer must initial that observation in 18a.

18b. ORGAN: Enter the organ that is considered abnormal. If an entry is made in this field at necropsy, then a corresponding "B" (observed) is to be entered in Field 17b. If the organ is found abnormal at trimming then the trimmer enters the appropriate information in Fields 18-22 and initials each new entry to the immediate left of Field 18a. If an entry is made in this field, an entry must also be made in at least one of the following fields: 20-"MORPHOLOGY", 21-"SIZE", or 22-"DISTRIBUTION and/or COLOR."

19. SITE: Enter the site within the organ that best describes the location of the abnormality. Multiple entries may be made in this field (ie., subcutis/r axilla). If no entry is made in the field, draw a short horizontal line indicating not applicable.

20. MORPHOLOGY: Enter the morphology observed in the organ. If more than one (1) morphologic observation is present in an organ, a separate entry must be made for each. If no entry is made in the field, draw a short horizontal line indicating not applicable.

COMMENT: Tissue NOS is the exception to the above statement. It is preferred that only one morphology is used. However, it may be necessary to use multiple morphologies to describe a large mass encompassing multiple organs (i.e., mass adhesion).

21. SIZE: Enter a numeric value to indicate the metric measurement of the organ or lesion and the unit of measurement. The measurements are recorded in 2 or 3 dimensions depending on the appropriateness. Quantity is an appropriate entry for fluids. If no entry is made in the field, draw a short horizontal line indicating not applicable.

22. DISTRIBUTION and/or COLOR: Enter the qualifier from the group-distribution and/or group-color that best describes the morphology of the organ. Multiple entries may be made in this field. If no entry is made in the field, draw a short horizontal line indicating not applicable.

23. TGL A: Refers to the gross TGL and is used to correlate the gross description with a microscopic diagnosis.

TGL B: The following are valid entries for TGL B:

1. The slide number(s) containing the microscopic lesion that correlate with the gross description listed in items 18-22 on the same line.
2. NCL - No Corresponding Lesion
3. NST - No Section Taken

NCL is used for those gross lesions in which there is no corresponding microscopic lesion, and no lesion found in the wet tissue or tissue block (ie. lymph node, mesenteric, enlarged, but normal microscopically). NST will be used for fluids, amputated tail, malocclusion of incisors or other lesions where no histologic section is prepared. All gross lesions will have a corresponding TGL slide number, NCL, or NST analog entered in TGL-B.

24. NOTES: Enter free text associated with the animal. The notes section is used for free text entered by the prosector, pathologist, or trimming technician during necropsy or trimming, and entries are to be initialed and dated. This section is not intended to be the only documentation of the presence of a lesion in an organ, but should be used to provide additional descriptive information or clarification of the appearance or location of a lesion.

Comments in the Notes section are to be preceded with the TGL number. Instances in which one topography was identified at necropsy, and another during the microscopic examination, are to be reconciled as in the following example: At necropsy a 2 cm diameter mass was observed in the inguinal skin (TGL-2); however, microscopically, it was determined to be a fibroadenoma of the mammary gland. The pathologist is to reconcile this by listing the TGL number and the correlate microscopic topography (i.e., TGL2-C-mammary gland, where C = correlates).

For the vast majority of TGLs, there is a corresponding lesion. In instances where the pathologist would have expected a microscopic lesion (ie. 2x3x5 mm skin mass), yet there was none, the block and wet tissues are to be examined for the "missing" lesion(s). If the lesion is located, obviously it is to be trimmed in and examined, and the slide number added to TGL-B. However, if after review of both the wet tissue and block, the lesion is not found, the pathologist is to indicate that the wet tissues and blocks were checked by including his/her initials and date in the Notes section corresponding to that TGL (i.e., TGL4 not observed).

25. PROSECTOR/ NECROPSY DATE: The prosector signs and dates the form at the completion of the necropsy.

26.	PATHOLOGIST/ NEC DATE:	The pathologist responsible for the necropsy of the animal signs and dates the form at the completion of the necropsy. The pathologist validates that the data are complete as soon after necropsy as possible even if he is not present for the performance of the necropsy.
27.	TRIMMER/DATE:	The trimming technician signs and dates Field 27 at completion of the trimming procedure.
28.	PROBABLE CAUSE OF MORIBUNDITY/ DEATH:	The pathologist enters the probable cause of death (PCOD) or cause of moribund condition at necropsy and initials and dates the entry. The pathologist is to address the PCOD based on the necropsy findings. It is not necessary for the pathologist to be present at the time of necropsy to determine the PCOD at necropsy. PCOD will be used for animals with a disposition of NATD, MSAC, DACC, and ACCK. In the case of moribund sacrifice animals, the cause of death will reflect the underlying cause of the moribund condition of the animal, NOT merely the reason for moribund sacrifice. If the PCOD/PCOM cannot be determined, then enter "undetermined".
29.	CLINICAL/ REMOVAL OBSERVATIONS:	Enter clinical removal observations, i.e., observations made at the time of removal of the animal from the study for all animals. In addition, enter the last formal clinical observations recorded in TDMS and the date of this last entry. At necropsy, the prosector is to indicate if they observed each of the listed clinical removal observations or not by entering a Y (yes) or N (no) and their initials next to each observation. This information is to aid the prosector and pathologist in observing, describing, and preserving lesions observed clinically. In addition, it aids the pathologist in interpreting and correlating gross and microscopic lesions.
30.	CORRECTION COMMENTS:	Enter in this field, the callout number, reason for the correction, initials, and date of correction.
	Page 1 of __ :	Fill in the total number of pages on the space provided. If more than 1 page is necessary number the pages consecutively.
	COMMENT:	The pathologist assigned to the necropsy is responsible for the conduct of the animal necropsy, proper collection and identification of tissues, and description of gross lesions using only the nomenclature in the PCT. The responsible pathologist who signs Field 26 should review header information at the top of the IANR for accuracy and make sure the prosector and trimmer complied with the requirements of the Specifications and the protocol. The assigned pathologist is responsible for providing the probable cause of death (PCOD/PCOM) in Field 28.

Individual Animal Necropsy Record - Continuation Page

The continuation page of the IANR is to be used when all of the gross abnormalities do not fit on the first page of the IANR. All header information must be completed. The continuation sheet is to be signed by the prosector and Pathologist (Fields 25 and 26) only if lesions listed on the continuation page were found at necropsy. The trimmer is to sign in Field 27.

This form must not be used without the completion of Page 1 of the IANR. It is not a stand-alone form. Refer to the previous section for a description of each field on this form.

INDIVIDUAL ANIMAL NECROPSY RECORD	1. FACILITY	2. TEST ARTICLE			3. PHASE
	Lab XX	SAMPLE			X CHRONIC
		4. DAYS ON TEST 692	5. DOSE 25 mg/Kg	6. ROUTE GV	SUBCHRONIC
					REPEATED DOSE

7. TDMS - NUMBER	8. SPECIES/STRAIN	9. SEX	10. ANIMAL NUMBER
9 9 9 9 9 - 0 5	RAT HSD	FEMALE	– – 2 9 8

11. HISTOLOGY NUMBER	12. DEATH DATE (MM/DD/YY)	13. BODY WEIGHT (G)
9 9 _ 9 9 9 9 _ 9 9 9	0 4 1 8 0 5	3 0 2 . 8

14. DISPOSITION	15. CONDITION	16a. IDENTIFICATION INFORMATION
TSAC / OTHER* / DACC	CANN/NTT	VERIFIED ☐ ID NUMBER: 2898
X MSAC / NATD / ACCK	X FRESH / PT CANN	IDENTIFICATION COMMENT:
SSAC	AUTO/TT / OTHER *	Should be 298; tattooed in error as 289; changed to 2898
* DEFINE OTHER:	* DEFINE OTHER:	16b. WET TISSUE REVIEW: X PERFORMED / NOT REQUIRED

17a. ORGAN	17b. NEC	17c. WGT (MG)	17a. ORGAN	17b. NEC	17c. WGT (MG)	17a. ORGAN	17b. NEC	17c. WGT (MG)	17a. ORGAN	17b. NEC	17c. WGT (MG)
ADRENAL GL.	A		JEJUNUM	A		MAMMARY GL	A		SALIVARY GLS	A	
BLOOD	A		ILEUM	A		MESENTERY	A		SKELETAL MUSCLE	A	
BLOOD VESSEL	A		INTEST LARGE			NERVE			SKIN	B	
BONE	A		CECUM	A		SCIATIC	A		SPINAL CORD	D	
BONE MARROW	A		COLON	A		TIBIAL	A		SPLEEN	~~A~~ [1] B	
BRAIN	A		RECTUM	A		TRIGEMINAL	A		STOMACH	A	
CARCASS	A		KIDNEY LEFT	A		NOSE	A		THYMUS	A	
CAVITIES	B		KIDNEY RIGHT	A		OVARY LEFT	A		THYROID GL	A	
CERVIX	A		LACRIMAL GL	A		OVARY RIGHT	A		TISSUE NOS		
CLITORAL GLAND	A		LARYNX	A		OVIDUCT	A		TONGUE	A	
ESOPHAGUS	A		LIVER	B		PANCREAS	A		TOOTH	A	
EYE	A		LUNG	B		PARATHYROID GL	A		TRACHEA	A	
HARDERIAN GL	A		LYMPH NODE			PHARYNX	A		URETER	A	
HEART	A		MANDIBULAR	A		PITUITARY GL	A		URINARY BLADDER	A	
INTEST SMALL			MESENTERIC	A					UTERUS	A	
DUODENUM	A		OTHER						VAGINA	A	
									ZYMBALS GL	A	

18a. TRIM ID	18b. ORGAN	19. SITE	20. MORPHOLOGY	21. SIZE	22. DISTRIBUTION / COLOR	23. TGL A	23. TGL B	24. NOTES
	Skin	Inguinal	Mass	80x40x40 mm	-	1	5	Liver contained > 5 lesions; five were entered on IANR M.D. 4/18/05
	Cavities	Chest	Fluid	1 ml	Red	2	NST	
	Spleen	-	Enlarged	30x20x20 mm	Dark	3	4	TGL 1-C-mammary gland PRM 10/7/05
	Lung	Left lobe	~~5x5x~~ [2] Nodule	5x5x5 mm	-	4	NCL	TGL 4 not observed PRM 10/7/05
	Liver	Median	Mass	15x10x10 mm	Dark	5	12	
	Liver	Median	Focus	3x3 mm	Dark	6	10	
	Liver	Left	Focus	4x4 mm	Dark	7	10	
L.S.	Small intestine	Duodenum	Nodule	2x2 mm	Hard, tan	8	10	
						9	10	
						10	13	

25. PROSECTOR/NECROPSY DATE	28. PROBABLE CAUSE OF MORIBUNDITY/DEATH:		30. CORRECTION COMMENTS:
R. Cutup 4/18/05	Liver tumor M.D. 4/18/05		[1] wrong entry RC 4/18/05
26. PATHOLOGIST/NEC DATE	29. CLINICAL/REMOVAL OBSERAVTIONS:	Y/N	[2] wrong place RC 4/18/05
M. Doctor 4/18/05	Thin	Y RC	
27. TRIMMER/DATE	Mass torso ventral large one	Y RC	
L. Slice 7/11/05	Lethargic LP 4/2/05 4/18/05	N RC	
A = NORMAL C = MISSING *B = OBSERVED D = PRESENT, NOT EXAMINED*	Abnormal breathing LP 4/18/05	N RC	PAGE 1 OF 1
REVISED 1/2011			

INDIVIDUAL ANIMAL NECROPSY RECORD	1. FACILITY	2. TEST ARTICLE			3. PHASE
					☐ CHRONIC
		4. DAYS ON TEST	5. DOSE	6. ROUTE	☐ SUBCHRONIC
					☐ REPEATED DOSE

7. TDMS – NUMBER	8. SPECIES/STRAIN	9. SEX	10. ANIMAL NUMBER
-	RAT HSD	MALE	

11. HISTOLOGY NUMBER	12. DEATH DATE (MM/DD/YY)	13. BODY WEIGHT (G)
		.

14. DISPOSITION	15. CONDITION	16a. IDENTIFICATION INFORMATION
☐ TSAC ☐ NATD ☐ DACC	☐ CANN/NTT	VERIFIED ☐ ID NUMBER:
☐ MSAC ☐ OTHER* ☐ ACCK	☐ FRESH ☐ PT CANN	**IIDENTIFICATION COMMENT:**
☐ SSAC	☐ AUTO/TT ☐ OTHER *	
* DEFINE OTHER:	* DEFINE OTHER:	**16b. WET TISSUE REVIEW** ☐ PERFORMED ☐ NOT REQUIRED

17a. ORGAN	17b. NEC	17c. WGT (MG)	17a. ORGAN	17b. NEC	17c. WGT (MG)	17a. ORGAN	17b. NEC	17c. WGT (MG)	17a. ORGAN	17b. NEC	17c. WGT (MG)
ADRENAL GL.			DUODENUM			OTHER			SEMINAL VESICLES		
BLOOD			JEJUNUM			MAMMARY GL			SKELETAL MUSCLE		
BLOOD VESSEL			ILEUM			MESENTERY			SKIN		
BONE			INTEST LARGE			NERVE			SPINAL CORD		
BONE MARROW			CECUM			SCIATIC			SPLEEN		
BRAIN			COLON			TIBIAL			STOMACH		
CARCASS			RECTUM			TRIGEMINAL			TESTIS LEFT		
CAVITIES			KIDNEY LEFT			NOSE			TESTIS RIGHT		
COAGULAT GL			KIDNEY RIGHT			PANCREAS			THYMUS		
COPER'S GLAND			LABC MUS. COM.			PARATHYROID GL			THYROID GL		
EPIDIDYMIS LEFT			LACRIMAL GL			PENIS			TISSUE NOS		
EPIDIDYMIS RIGHT			LARYNX			PHARYNX			TONGUE		
ESOPHAGUS			LIVER			PITUITARY GL			TOOTH		
EYE			LUNG			PREPUTIAL GL			TRACHEA		
HARDERIAN GL			LYMPH NODE			PROSTATE, VENT			URETER		
HEART			MANDIBULAR			PROS, DORS-LAT			URINARY BLADDER		
INTEST SMALL			MESENTERIC			SALIVARY GLS			ZYMBALS GL		

18a. TRIM ID	18b. ORGAN	19. SITE	20. MORPHOLOGY	21. SIZE	22. DISTRIBUTION / COLOR	23. TGL A	23. TGL B	24. NOTES
						1		
						2		
						3		
						4		
						5		
						6		
						7		
						8		
						9		
						10		

25. PROSECTOR/NECROPSY DATE	28. PROBABLE CAUSE OF MORIBUNDITY/DEATH:		30. CORRECTION COMMENTS:
PATHOLOGIST/NEC DATE	**29. CLINICAL/REMOVAL OBSERAVTIONS:**	**Y/N**	
TRIMMER/DATE			
A = NORMAL C = MISSING			
B = OBSERVED D = PRESENT, NOT EXAMINED			
REVISED 1/2011			**PAGE 1 OF _____**

INDIVIDUAL ANIMAL NECROPSY RECORD	1. FACILITY	2. TEST ARTICLE			3. PHASE
					☐ CHRONIC
		4. DAYS ON TEST	5. DOSE	6. ROUTE	☐ SUBCHRONIC
					☐ REPEATED DOSE

7. TDMS - NUMBER	8. SPECIES/STRAIN	9. SEX	10. ANIMAL NUMBER
-	RAT HSD	FEMALE	

11. HISTOLOGY NUMBER	12. DEATH DATE (MM/DD/YY)	13. BODY WEIGHT (G)
		.

14. DISPOSITION

☐ TSAC ☐ OTHER* ☐ DACC
☐ MSAC ☐ NATD ☐ ACCK
☐ SSAC

* DEFINE OTHER:

15. CONDITION

☐ FRESH ☐ CANN/NTT
☐ AUTO/TT ☐ PT CANN
☐ OTHER *

* DEFINE OTHER:

16a. IDENTIFICATION INFORMATION

VERIFIED ☐ ID NUMBER:

IDENTIFICATION COMMENT:

16b. WET TISSUE REVIEW ☐ PERFORMED ☐ NOT REQUIRED

17a. ORGAN	17b. NEC	17c. WGT (MG)	17a. ORGAN	17b. NEC	17c. WGT (MG)	17a. ORGAN	17b. NEC	17c. WGT (MG)	17a. ORGAN	17b. NEC	17c. WGT (MG)
ADRENAL GL.			JEJUNUM			MAMMARY GL			SALIVARY GLS		
BLOOD			ILEUM			MESENTERY			SKELETAL MUSCLE		
BLOOD VESSEL			INTEST LARGE			NERVE			SKIN		
BONE			CECUM			SCIATIC			SPINAL CORD		
BONE MARROW			COLON			TIBIAL			SPLEEN		
BRAIN			RECTUM			TRIGEMINAL			STOMACH		
CARCASS			KIDNEY LEFT			NOSE			THYMUS		
CAVITIES			KIDNEY RIGHT			OVARY LEFT			THYROID GL		
CERVIX			LACRIMAL GL			OVARY RIGHT			TISSUE NOS		
CLITORAL GLAND			LARYNX			OVIDUCT			TONGUE		
ESOPHAGUS			LIVER			PANCREAS			TOOTH		
EYE			LUNG			PARATHYROID GL			TRACHEA		
HARDERIAN GL			LYMPH NODE			PHARYNX			URETER		
HEART			MANDIBULAR			PITUITARY GL			URINARY BLADDER		
INTEST SMALL			MESENTERIC						UTERUS		
DUODENUM			OTHER						VAGINA		
									ZYMBALS GL		

18a. TRIM ID	18b. ORGAN	19. SITE	20. MORPHOLOGY	21. SIZE	22. DISTRIBUTION / COLOR	23. TGL A	23. TGL B	24. NOTES
						1		
						2		
						3		
						4		
						5		
						6		
						7		
						8		
						9		
						10		

25. PROSECTOR/NECROPSY DATE	28. PROBABLE CAUSE OF MORIBUNDITY/DEATH:		30. CORRECTION COMMENTS:
26. PATHOLOGIST/NEC DATE	29. CLINICAL/REMOVAL OBSERAVTIONS:	Y/N	
27. TRIMMER/DATE			
A = NORMAL *C = MISSING* *B = OBSERVED* *D = PRESENT, NOT EXAMINED*			
REVISED 1/2011			**PAGE 1 OF _____**

INDIVIDUAL ANIMAL NECROPSY RECORD	1. FACILITY	2. TEST ARTICLE			3. PHASE
					☐ CHRONIC
		4. DAYS ON TEST	5. DOSE	6. ROUTE	☐ SUBCHRONIC
					☐ REPEATED DOSE

7. TDMS - NUMBER	8. SPECIES/STRAIN	9. SEX	10. ANIMAL NUMBER
-	MOUSE CD 1	MALE	

11. HISTOLOGY NUMBER	12. DEATH DATE (MM/DD/YY)	13. BODY WEIGHT (G)
		.

14. DISPOSITION	15. CONDITION	16a. IDENTIFICATION INFORMATION
☐ TSAC ☐ OTHER* ☐ DACC	☐ CANN/NTT	VERIFIED ☐ ID NUMBER:
☐ MSAC ☐ NATD ☐ ACCK	☐ FRESH ☐ PT CANN	**IDENTIFICATION COMMENT:**
☐ SSAC	☐ AUTO/TT ☐ OTHER *	
* DEFINE OTHER:	* DEFINE OTHER:	**16b. WET TISSUE REVIEW** ☐ PERFORMED ☐ NOT REQUIRED

17a. ORGAN	17b. NEC	17c. WGT (MG)	17a. ORGAN	17b. NEC	17c. WGT (MG)	17a. ORGAN	17b. NEC	17c. WGT (MG)	17a. ORGAN	17b. NEC	17c. WGT (MG)
ADRENAL GL.			INTEST SMALL			LYMPH NO, OTHER			SKELETAL MUSCLE		
BLOOD			DUODENUM			MAMMARY GL			SKIN		
BLOOD VESSEL			JEJUNUM			MESENTERY			SPINAL CORD		
BONE			ILEUM			NERVE			SPLEEN		
BONE MARROW			INTEST LARGE			SCIATIC			STOMACH		
BRAIN			CECUM			TRIGEMINAL			TESTIS LEFT		
CARCASS			COLON			NOSE			TESTIS RIGHT		
CAVITIES			RECTUM			PANCREAS			THYMUS		
COAGULAT GL			KIDNEY LEFT			PARATHYROID GL			THYROID GL		
COPER'S GLAND			KIDNEY RIGHT			PENIS			TISSUE NOS		
EPIDIDYMIS LEFT			LACRIMAL GL			PHARYNX			TONGUE		
EPIDIDYMIS RIGHT			LARYNX			PITUITARY GL			TOOTH		
ESOPHAGUS			LIVER			PREPUTIAL GL			TRACHEA		
EYE			LUNG			PROSTATE, VENT			URETER		
GALLBLADDER			LYMPH NODE			PROST, DORS-LAT			URINARY BLADDER		
HARDERIAN GL			MANDIBULAR			SALIVARY GLS			ZYMBALS GL		
HEART			MESENTERIC			SEMINAL VESICLE					

18a. TRIM ID	18b. ORGAN	19. SITE	20. MORPHOLOGY	21. SIZE	22. DISTRIBUTION / COLOR	23. TGL A	23. TGL B	24. NOTES
						1		
						2		
						3		
						4		
						5		
						6		
						7		
						8		
						9		
						10		

25. PROSECTOR/NECROPSY DATE	28. PROBABLE CAUSE OF MORIBUNDITY/DEATH:		30. CORRECTION COMMENTS:
26. PATHOLOGIST/NEC DATE	29. CLINICAL/REMOVAL OBSERAVTIONS:	Y/N	
27. TRIMMER/DATE			
A = NORMAL C = MISSING			
B = OBSERVED D = PRESENT, NOT EXAMINED			
REVISED 1/2011			**PAGE 1 OF _____**

INDIVIDUAL ANIMAL NECROPSY RECORD	1. FACILITY	2. TEST ARTICLE			3. PHASE
					☐ CHRONIC
		4. DAYS ON TEST	5. DOSE	6. ROUTE	☐ SUBCHRONIC
					☐ REPEATED DOSE

7. TDMS - NUMBER	8. SPECIES/STRAIN	9. SEX	10. ANIMAL NUMBER
-	MOUSE CD 1	FEMALE	

11. HISTOLOGY NUMBER	12. DEATH DATE (MM/DD/YY)	13. BODY WEIGHT (G)
		.

14. DISPOSITION

☐ TSAC ☐ OTHER* ☐ DACC
☐ MSAC ☐ NATD ☐ ACCK
☐ SSAC

* DEFINE OTHER:

15. CONDITION

☐ CANN/NTT
☐ FRESH ☐ PT CANN
☐ AUTO/TT ☐ OTHER *

* DEFINE OTHER:

16a. IDENTIFICATION INFORMATION

VERIFIED ☐ ID NUMBER:

IDENTIFICATION COMMENT:

16b. WET TISSUE REVIEW ☐ PERFORMED ☐ NOT REQUIRED

17a. ORGAN	17b. NEC	17c. WGT (MG)	17a. ORGAN	17b. NEC	17c. WGT (MG)	17a. ORGAN	17b. NEC	17c. WGT (MG)	17a. ORGAN	17b. NEC	17c. WGT (MG)
ADRENAL GL.			INTEST SMALL			LYMPH NODE			SALIVARY GLS		
BLOOD			DUODENUM			MANDIBULAR			SKELETAL MUSCLE		
BLOOD VESSEL			JEJUNUM			MESENTERIC			SKIN		
BONE			ILEUM			OTHER			SPINAL CORD		
BONE MARROW			INTEST LARGE			MAMMARY GL			SPLEEN		
BRAIN			CECUM			MESENTERY			STOMACH		
CARCASS			COLON			NERVE			THYMUS		
CAVITIES			RECTUM			SCIATIC			THYROID GL		
CERVIX			KIDNEY LEFT			TRIGEMINAL			TISSU E NOS		
CLITORAL GL			KIDNEY RIGHT			NOSE			TONGUE		
ESOPHAGUS			LACRIMAL GL			OVARY LEFT			TOOTH		
EYE			LARYNX			OVARY RIGHT			TRACHEA		
GALLBLADDER			LIVER			OVIDUCT			URETER		
HARDERIAN GL			LUNG			PANCREAS			URINARY BLADDER		
HEART						PARATHYROID GL			UTERUS		
						PHARYNX			VAGINA		
						PITUITARY GL			ZYMBALS GL		

18a. TRIM ID	18b. ORGAN	19. SITE	20. MORPHOLOGY	21. SIZE	22. DISTRIBUTION / COLOR	23. TGL A	23. TGL B	24. NOTES
						1		
						2		
						3		
						4		
						5		
						6		
						7		
						8		
						9		
						10		

25. PROSECTOR/NECROPSY DATE	28. PROBABLE CAUSE OF MORIBUNDITY/DEATH:		30. CORRECTION COMMENTS:
26. PATHOLOGIST/DATE	29. CLINICAL/REMOVAL OBSERAVTIONS:	Y/N	
27. TRIMMER/DATE			
A = NORMAL C = MISSING *B = OBSERVED D = PRESENT, NOT EXAMINED*			
REVISED 1/2011			**PAGE 1 OF _____**

INDIVIDUAL ANIMAL NECROPSY RECORD	1. FACILITY	2. TEST ARTICLE			3. PHASE
					☐ CHRONIC
		4. DAYS ON TEST	5. DOSE	6. ROUTE	☐ SUBCHRONIC
					☐ REPEATED DOSE

7. TDMS - NUMBER	8. SPECIES/STRAIN	9. SEX	10. ANIMAL NUMBER

11. HISTOLOGY NUMBER

CONTINUATION PAGE

18a. TRIM ID	18b. ORGAN	19. SITE	20. MORPHOLOGY	21. SIZE	22. DISTRIBUTION /COLOR	23. TGL A	23. TGL B	24. NOTES
						11		
						12		
						13		
						14		
						15		
						16		
						17		
						18		
						19		
						20		
						21		
						22		
						23		
						24		
						25		

25. PROSECTOR/NECROPSY DATE	30. CORRECTION COMMENTS:
26. PATHOLOGIST/DATE	
27. TRIMMER/DATE	
A = NORMAL C = MISSING B = OBSERVED D = PRESENT, NOT EXAMINED	
REVISED 1/2011	PAGE 2 OF _____

APPENDIX 5 - Procedures for Sperm Motility & Counts and Vaginal Cytology Evaluation (SMCVCE)

I. MALE ORGAN TOXICITY EVALUATION IN MICE AND RATS

The NTP toxicology laboratories are urged to read the material thoroughly because there are a few key differences between mouse and rat studies. Sperm motility is highly dependent on having available energy substrate (a buffer containing lactate and glucose), pH (should be 7.25 $\pm$ 0.05) and temperature (with rapid decrease in motility with only a few degrees C drop in temperature). Thus all buffers and equipment should be temperature controlled or be held in an incubator or on a slide warmer at 37°C during sample preparation and evaluation. Moreover, rodent sperm are likely to form aggregates that prohibit appropriate motility analysis (a single cell suspension is required) and for this reason a protein supplement is normally added to the buffer, frequently 1% Bovine Serum Albumin (BSA) or egg yolk in modified Tyrode's buffer.

The conduct of sperm motility assessment and sperm count may be performed using manual or automated procedures. Although the automated procedure is preferred, manual assessment will be accepted. Procedures for both methods are provided in this document.

A. SUGGESTED LIST OF MATERIALS & SUPPLIES FOR SPERM ASSAYS IN MICE AND RATS

To be supplied by the toxicology testing laboratory

NOTE:
Materials & supplies needed will be based on the Method used – Automated versus Manual.

- Scalpels, scissors, forceps, syringes
- Pipettes (1.0 and 5.0 mL), pipettors and tips
- Pre-cleaned microscope slides (3" x 1") and cover-slips (24 x 40 mm)
- Microhematocrit capillary tubes
- Test tubes (16 x 100 mm), test tube racks
- Labels
- Test Yolk Buffer
- Incubator, water bath, slide warmer
- Timers
- pH meter and pH paper
- Oven
- CASA
- Pasteur pipettes
- Petri plates (15 x 60 mm)
- Parafilm
- Hemacytometers and cover-slips (improved Neubaur, 0.1 mm deep)
- Scintillation Vials 28 x 61 mm or Sample Vials 28 x 60 mm and Scintillation Vial Racks
- Modified Tyrode's Solution
- Phosphate Buffered Saline
- Hand tally counter (ENM-4290 or Clay Adams 4300)
- Stage warmer for the microscope
- Thermometers
- Vortex
- Protective Clothing

B. PREPARATION OF BUFFER/STAIN FOR SPERM ASSAYS

1. **Buffer for Automated Mouse and Rat Method**

The diluent (Modified Tyrode's Solution) can be prepared (See below) or obtained from the NTP SCVCE contractor through the Project Officer.

To make 1000 mL of Tyrode's solution, bring the following up to 1000 mL with distilled deionized water:

Ingredients	Buffer Concentration	Amount/Liter
NaCl	99.23 mM	5.801 g/L
KCl	2.68 mM	0.200 g/L
$CaCl_2.2H_20$	1.80 mM	0.265 g/L
$NaH_2PO_4.H_20$	0.36 mM	0.050 g/L
$MgCl_2.6H_20$	0.49 mM	0.100 g/L
$NaHC0_3$	25.00 mM	2.1 g/L
Na lactate	25.00 mM	2.801 g/L
Na pyruvate	0.50 mM	0.055 g/L
Glucose	5.56 mM	1.001 g/L

Mix thoroughly until all chemicals are properly dissolved.

To the buffer, add 10 mL of Bovine Serum Albumin (BSA) to make a 1% solution. Shake well until in solution. Adjust the pH to 7.2 with 1N HCl or 1N NaOH. Aliquot the buffer into 5 or 10 mL volumes.

Store at -20^{o}C. Prior to each usage, the buffer's pH should be taken and adjusted to 7.2 if needed. If the buffer is kept at 37^{o}C (incubator temperature) for long periods of time (1-2 hours) then again the pH should be taken and adjusted if necessary.

2. **Buffer for Manual Mouse Method**

The diluent (Modified Tyrode's Solution) can be prepared (See below) or obtained from the NTP SCVCE contractor through the Project Officer.

To make 1000 mL of Tyrode's solution, bring the following up to 1000 mL with distilled deionized water:

Ingredients	Buffer Concentration	Amount/Liter
NaCl	99.23 mM	5.801 g/L
KCl	2.68 mM	0.200 g/L
$CaCl_2.2H_20$	1.80 mM	0.265 g/L
$NaH_2PO_4.H_20$	0.36 mM	0.050 g/L
$MgCl_2.6H_20$	0.49 mM	0.100 g/L
$NaHC0_3$	25.00 mM	2.1 g/L
Na lactate	25.00 mM	2.801 g/L
Na pyruvate	0.50 mM	0.055 g/L
Glucose	5.56 mM	1.001 g/L

Mix thoroughly until all chemicals are properly dissolved.

To the buffer, add 200 mL of egg yolk without the yolk membranes. Shake until yolk is in solution. Thermoprecipitate the solution by boiling for 3 minutes at 92-95^{o}C. Filter through 4 layers of cheesecloth. Centrifuge the filtrate at 10,000 rpm for 20 minutes. Filter through Whatman #1 paper. Add 0.7 g/100 mL of percent of D-anhydrous glucose to the solution to raise the osmolarity. Measure the osmotic pressure and it should be at 325 mOsm/L (accuracy within 5 mOsm/L). Adjust the pH to 7.2 with 1N HCl or 1N NaOH. At this time add penicillin (100 u/mL) and streptomycin (50 ug/mL). Aliquot the buffer into 5 or 10 mL volumes. Since this buffer is easily contaminated, it is recommended that all steps after thermoprecipitation be conducted under aseptic atmosphere when possible.

Store at -20°C. Prior to each usage, the buffer's pH should be taken and adjusted to 7.2 if needed. If the buffer is kept at 37°C (incubator temperature) for long periods of time (1-2 hours) then again the pH should be taken and adjusted if necessary.

3. **Buffer and Stain for Manual Rat Method**

a. Test Yolk Buffer can be obtained from the NTP contractor for SCVCE studies(request is made through the Project Officer), or prepared using the following ingredients per liter:

TES**	14.5 g
TRIS***	3.5 g
Glucose	2.0 g

**(N-tris(hydroxymethly)methyl-1-aminoethane sulfonic acid)
***Tris(hydroxymethly)aminomethane

Skim Milk Powder	33.0 g
Sodium Citrate	6.0 g

Mix the ingredients in 700 mL of distilled deionized water. Adjust the final volume to 800 mL. Add 200 mL of egg yolk without the yolk membranes. Thermoprecipitate the solution by boiling at 92-95^{o}C for 3 minutes. Filter through 4 layers of cheesecloth. Centrifuge the filtrate at 10,000 rpm for 20 minutes. Filter through Whatman #1 paper. Aliquot into 5 or 10 mL volumes. Store at -20^{o}C until further use. pH will be 7.1-7.2.

4. **Phosphate Buffered Saline (PBS)**

PBS is prepared by using the following ingredients per 1000 mL:

NaCl	8.0 g
KCl	0.2 g
KH_2PO_4	0.2 g
$Na_2HPO_47H_2O$	2.1 g
$CaCl_2$	0.1 g
Mg Cl_26H_2	0.1 g

Final pH = 7.2

Mix the contents in 950 mL of distilled deionized water. Adjust the pH to 7.2. Bring the final volume to 1000 mL. Filter sterilization is recommended.

C. PROCEDURES FOR REMOVING AND WEIGHING TISSUES

Before euthanizing the animal, be sure that the buffer solution, microscope slides, cover-slips, pipette tips, petri plates, and microscope stage have been warmed to the appropriate temperature (37°C). Also, be sure that the balance is properly calibrated. Check the pH of the appropriate buffer solution and adjust it to 7.2 with HCL (1N) or NaOH (1N).

NOTE: For the Automated Method, use buffer on page 6 for both mice and rats.
For the Manual Method, use the formula on page 7 for mice and page 8 for rats.

The test animal is weighed, (the weight is recorded on the Data Sheet in this Appendix, with separate pages for mice or rats) and then euthanized according to the study protocol at the designated time, typically at the end of a 90-day study.

1. Procedure for Removing Testis and Epididymis

After euthanizing the animal, conduct the sperm collection as quickly as possible. Use the following procedure to remove the testis and epididymis:

a. Pull the left testis into the abdominal cavity by grasping the fat surrounding the caput epididymis. Excise the left testis by cutting the vas deferens approximately 0.5 cm from the point of attachment of the cauda epididymis. The caput epididymis should be located prior to excision so that it will not be clipped accidentally. See Section F. Figures 1 (mouse), 2 (rat), and 3 of this Appendix.

b. The intact left epididymis is removed and weighed to the nearest 0.0001 g. Record the weight on Data Sheet.

c. Clip the left cauda epididymis at the point of origin of the ductus deferens at the distal end and at the boundary between the distal corpus and cauda epididymis of the proximal end. In rats, cauda epididymis can be seen as white tissue consisting of convoluted tubules.

d. Weigh left cauda epididymis to the nearest 0.0001 g. Record the weight on Data Sheet.

2. Weighing, Freezing and Shipping of Testis

a. During the incubation period for the motility assessment, the left testis is to be weighed and frozen for testicular spermatid head-count as described below.

Label each vial (preferably plastic) with the appropriate laboratory name, test article, route, dose group, animal number, L for left testis (or R for right testis when required), and date of collection.

Weigh the left testis and record the weight to 0.0001 g on Data Sheet.

Place the testis in the appropriately labeled vial and freeze on dry ice.

Transfer frozen samples to a freezer (-70 to -80°C) for storage until ready for shipment to NTP contractor for SCVCE studies.

b. Instructions for shipping frozen testis to SCVCE laboratory

The NTP toxicology testing laboratory conducting the toxicity studies is responsible for packaging and shipping costs.

The frozen testes shall be shipped to the NTP-designated SCVCE laboratory along with the corresponding raw data sheets within two weeks of necropsy.

All vials shall be placed in vial racks and wrapped with bubble packing to avoid breakage during shipment.

The wrapped vial racks shall be placed in 350 lb. styrofoam-lined boxes filled with dry ice for shipment.

An inventory of the vials shall be sent with the cover letter.

Shipping cartons shall be sealed and bound with filament tape prior to shipment and labeled "freeze upon arrival".

Shipping shall be expedited so that the testes do not thaw during transit, i.e. overnight delivery. The laboratory must be notified prior to shipment so that they will anticipate delivery.

D. AUTOMATED METHOD FOR SPERM MOTILITY ASSESSMENT AND COUNT

Most CASA (Computerized Assisted Sperm Analysis) machines (the Hamilton Thorne IVOS Tox is recommended) require that the sperm samples be appropriately diluted in buffer before a sample is taken for analysis to allow conditions for uninhibited sperm motion. This can be determined experimentally but is usually approximately 50 mL per cauda for rats and 10 mL per cauda for mice. Other methods of dilution (e.g. a serial dilution from 10 mL mixed with a rat cauda) are acceptable if they give consistent results and concentrations in the CASA chamber that do not impede normal motion (< 100 but > 50 sperm per field). See manufacturer's instructions for more detail.

1. Processing Tissues

Prepare pre-warmed Petri dishes labeled for the individual animal containing an appropriate volume of buffer containing BSA.

Isolate and weigh the cauda as per method described above, place in a Petri dish containing the appropriate buffer and make 2 to 3 small pin-point incisions with a scalpel blade into the distal tip of the proximal cauda epididymis (see Section F. Figure 3). Replace Petri lid and incubate for 2.5 minutes at 37°C. After incubation gently swirl the Petri dish to mix the sperm suspension. Load slide/chamber for the CASA instrument (by capillary action) and then follow the CASA manufacturer's operating instructions to obtain motility and count data. The machine will enable the collection of an individual animal number (as a file number), the dilution undertaken and the weight of the cauda.

2. Sperm Motility Measurements

Check to assure the dilution is appropriate by manually scanning fields on the machine (there should be no more than ~100 sperm per field) and either collect data or further dilute the sample appropriately. Acquire motility and motion characteristics as per manufacturer's instructions. Acquire images from 5 different fields and save to a suitable disk for the capture of electronic data (motion data should be from at least 250 sperm). For samples with low counts, more fields may have to be taken to obtain the requisite 250 sperm. All the motion characteristics should be saved (as raw data) to a disk. If necessary, these images can be re-analyzed at a future date.

The CASA machine will provide the following data that can be exported and saved in a suitable electronically readable file (e.g. ASCII) for further statistical analysis:

Counts	-	Total sperm, Motile sperm, and Progressively motile sperm
		% Motile and % Progressively motile
		Slow cells and Static cells
Concentration	-	Total, Motile, Progressively motile (in millions/mL)
		Slow cells and Static cells (millions/mL)

Other velocity parameters are available from the CASA and can be useful and sensitive indicators of change indicating an adverse quantitative effect on sperm motility/motion (as opposed to the qualitative manual method). Such measurements are made automatically by the instrument and should be retained as part of the dataset for each animal:

VAP - smooth path velocity, microns/sec
VCL - track velocity, microns/sec (sometimes called curvilinear velocity where computer "smoothes" the sperm track to obtain velocity over the whole period of measurement)
VSL - straight-line velocity, microns/sec

3. Sperm Count

With the "ident" mode on the Hamilton Thorne machine, a sample may be taken, after motility analysis, of the total caudal count by gently mincing the cauda in buffer with fine scissors. Following addition of the manufacturer's stain and suitable dilution, (see manufacturer's instructions) sperm numbers can be obtained from the cauda on a million/gram of tissue basis.

4. Data Submission

Complete the top portion of the Data Sheet (separate pages for mice and rats) and check the box to indicate that sperm motility and count were performed using AUTOMATED MEASUREMENTS (leave the bottom portion of the Data Sheet blank since these measurements will be generated by CASA). Submit a hard copy of all of the sperm count, concentration and velocity data for each animal, together with a suitable electronic form of the data on disk for statistical analysis. In addition, raw motion images shall be saved electronically to disk for each study. All of this information, along with the Data Sheets, is to be sent to the NTP-designated SCVCE contractor for evaluation.

E. MANUAL METHOD FOR SPERM MOTILITY ASSESSMENT AND COUNT

1. Processing Tissues. - **This step differs for mice and rats. Read instructions carefully.**

Mice

a. Two independent slides are prepared to estimate sperm motility. Place < 100 μL of pre-warmed modified Tyrode's Solution (see page 6) on each microscope slide. Slides shall be placed on a slide warmer set at 37°C and retained at this temperature.

b. Hold the left cauda epididymis with forceps and make a small incision near the center of the cauda with a sharp, clean surgical blade. See Section F. Figure 4.

c. Apply gently pressure on the cauda surface so that a small epididymal tubule is exposed or caudal fluid begins to ooze out into the Tyrode's solution on the pre-warmed slide (Caution: Do not apply too much pressure since there is an ample amount of sperm in a piece of cauda equivalent to 1/2 of a pinhead. Use of excessive amounts of cauda fluid for the sperm motility counts may significantly lower sperm density evaluations and impede your determination of sperm motility). Alternately, the cauda epididymis is placed into a petri plate (15 x 60 mm) containing 2 mL of pre-warmed phosphate buffered saline (PBS). The cauda is then held in place with forceps and a very small piece (not more than 1/20 of the total caudal is sliced off and the cut surface applied briefly to the buffer (see Section F. Figure 4). Note: Surgical blades must be cleaned with saline between animals.

d. If necessary, the cauda fluid may be further chopped on the slides with a pair of needles or sharp blades.

e. Place a warm cover-slip (24 x 40 mm) on the slide.

Maximum sperm motility is obtained if the removal of cauda, weighing, and preparation of sperm suspension is done as quickly as possible (approximately 2-4 minutes).

f. Two slides are prepared simultaneously for estimation of sperm motility.

g. The sperm motility is estimated without any further delay as illustrated in Section 2 below.

h. Place remaining caudal tissue into a petri plate (15 x 60 mm) containing 2 mL of phosphate buffered saline (PBS).

i. The remaining caudal tissue is gently chopped (only after the sperm motility counts have been performed) in the petri plate with two scalpels until its contents are released. The petri plate is swirled several times and incubated for 15 minutes at 37°C. It allows aggregates of sperm, which can be seen as pieces of tissue in the suspension, to break up. Petri plates should remain covered during the incubation period.

Rats

a. The cauda epididymis in rats can be seen as white tissue consisting of convoluted tubules. After clipping the cauda at the point of origin of the ductus deferens (Section F. Figures 2, and 3) from the cut end of the cauda (distal cauda), a very small sperm sample (not greater than the size of a pinhead) is removed with the tip of the scalpel blade and placed on a pre-warmed slide with < 100 uL drops of test yolk buffer (see Section B) at 37°C. Do not allow the sperm to dry on the scalpel blade.

b. A cover-slip (24 x 40 mm) is placed on the slide after gentle mixing of sperm in the buffer solution. Two such slides are prepared for estimation of sperm motility as described in Section 2 below.

c. After motility has been estimated, the remaining cauda is weighed to the nearest 0.0001 g. The weight is recorded on the Data Sheet.

d. This step is performed only after sperm motility has been estimated. The cauda is held in place with a scalpel and gently chopped with another sharp scalpel in 10 mL of 0.9% saline or PBS in a petri plate incubated at 37^{o}C for 15 minutes.

2. Estimation of Sperm Motility - **this step is common for both rats and mice.**

a. To estimate sperm motility, the viewer counts the actual number of motile and non-motile sperm for each field of vision. A small hand counter may be used to count the number of motile and non-motile sperm. No more than 20 sperm should be in the visual field.

b. The final motility is based on ten independent fields and observations made by two independent viewers.

c. Person A evaluates the motility in five independent fields and similarly, Person B evaluates the motility for another five independent fields. Both viewers must use independent slides and when possible, independent microscopes.

d. Sperm are considered motile if they show any movement at all. A 40X objective is recommended to evaluate sperm motility.

e. Percent motility decreases with time; therefore, evaluation is critical and must be accomplished as quickly as possible (2-4 minutes).

f. A microscope stage warmer (37°C) is needed for sperm motility evaluation and to maintain the sperm suspension at optimum temperature.

Sperm are very sensitive to temperature and pH changes. Extreme care must be taken to keep all materials at 37°C on a slide warmer and/or in an incubator. The pH of the Tyrode's solution should be checked routinely and adjusted when and if necessary. These factors are very important to obtain an accurate estimation of sperm motility.

g. Average sperm motility readings should be approximately 75% or higher in control mice and rats. If sperm motility is considerably lower than 75%, there is some problem and the Project Officer and personnel at the NTP contractor for SCVCE studies shall be consulted.

3. Sperm Count

After the 15-minute incubation period, a sperm count is performed but the sperm suspension must be further diluted. Due to a significant difference in the cauda weight for rats and mice, parts of the dilution procedures for the two species are different and should be noted.

a. Swirl the petri plate to evenly distribute the sperm. In the event the sperm suspension is not uniform, gently chop the cauda until a uniform suspension is obtained, incubate further if necessary. Transfer 0.5 mL (using a 1.0 mL pipette) to a test tube (16 x 100 mm) containing 2 mL of the phosphate buffered saline. The rat suspension should be gently pipetted 20 times to break up sperm aggregates.

b. After transferring 0.5 mL of sperm suspension into 2 mL of PBS, transfer 2 aliquots of the residual suspension into two 1.5 mL vials (preferably plastic, filled to approximately 80%) and place on dry ice. These samples are to be shipped to the NTP Archives following the shipping instructions provided above for testes.

c. Gently and thoroughly mix the suspension with a Vortex mixer for 2 seconds. If a vortex mixer is not available, gently agitate the sperm suspension then gently pipette (with a Pasteur pipette) approximately 5 times to thoroughly mix the suspension. The importance of thorough mixing cannot be overstated.

d. The volume of PBS may be varied according to the density of sperm suspension. The number of sperm per secondary square (see Step g. below) should not be less than 5 or more than 15.

e. The volume of sperm suspension and PBS solution used for the sperm count shall be recorded on the data form if different from the suggested volumes.

f. The sperm used for this assay shall be killed by placing the test tube in hot water for one minute, in an ice bath for 5 minutes, or adding formaldehyde or glutaraldehyde to 0.5% final concentration.

g. The sperm suspension is thoroughly mixed by vortexing $\geq$ 5 seconds, and placed on a hemacytometer (see Section F. Figure 5).

h. The shiny surfaces in the middle of the hemacytometer have microscopic grids consisting of heavy lines which divide the field into 25 large squares. Within each of the large squares are 16 smaller squares. The cover-slip does not touch the grid area when placed over the middle portion.

i. Microhematocrit capillary tubes are used to place suspension on the hemacytomer. The sperm suspension is placed on the hemacytometer by touching the tip of the capillary tube to the angle formed by the cover-slip and counting chamber of the hemacytometer. The area under the cover-slip should not be overfilled.

j. Two counts are performed using separate samples of the diluted suspension. Both slides for sperm count must be prepared after thorough mixing of the sperm suspension. Consistency in the preparation of the sperm samples for density determinations is essential to obtain accurate results.

k. To perform the counts, the counting area is located under low power (10X objective) and then switched to the 20X objective for counting. It is not necessary to count every cell in every square. The number of sperm in five secondary squares is counted. Sperm lying on the lines on the bottom and right sides of the large squares are included so that they will not be counted twice.

l. In case there are less than five (5) sperm per secondary square and it is not possible to make a new (less diluted) suspension, count sperm in two (2) tertiary squares. This change must be recorded on the Data Sheet and taken into consideration while estimating sperm density.

m. Calculation for determining sperm density also varies for mice and rats as explained below:

- The viewer has counted the sperm in 1/50 mm^3 volume;
- The dilution factor for the caudal sperm is the final dilution of caudal weight (normally for mice it is 10 cc/caudal weight, and for rats 50 cc/caudal weight). These values apply only when the suggested volumes are used.
- The number of cells counted multiplied by the volume fraction (1/50 mm^3) counted multiplied by the dilution factor should equal the number of cells per cubic millimeter.
- Sperm concentration is normally expressed as number of sperm per gram of tissue weight. Sperm density in the control mice is generally between 500 x 10^6 and 1000 x 10^6 sperm per gram caudal tissue.

4. Calculation Procedure For Estimation Of Sperm Density

Mice

Suppose the mean number of sperm in "5" secondary squares in mice = 20. The number of sperm in 2 X 10^{-5} mL of diluted sperm suspension = 20. The number of sperm per mL of diluted suspension =

$$\frac{20}{2 \times 10^{-5}} = 1 \times 10^6/\text{mL}$$

Since the diluted suspension represents one-fifth of the original suspension, if the standard dilution of 0.5 mL in 2 mL was used, the number of sperm per mL of the original suspension =

5 X (1 X 10^6) = 5 X 10^6.

Volume of the original suspension = 2 mL.

Total sperm in the original suspension = 2 X (5 X 10^6) = 1 X 10^7/suspension = number sperm/cauda.

If cauda weight = 0.03000 g, total sperm in 0.03000 g of caudal tissue = 1 X 10^7.

Total sperm per "g" of caudal tissue =

$\frac{1 \times 10^7}{0.0300} = 3.33 \times 10^8$ sperm/g cauda.

This value can also be estimated by the following formula:

$$\frac{\text{Mean No. of sperm in 5 secondary squares}}{\text{caudal weight in g}} \times 50{,}000 \times 10 \text{ (dilution factor)}$$

When recording final calculated number of sperm/g cauda, record this to one decimal place.

Rats

Suppose the mean number of sperm in "5" secondary squares in mice = 20. The number of sperm in 2×10^{-5} mL of diluted sperm suspension = 20. The number of sperm per mL of diluted suspension =

$$\frac{20}{2 \times 10^{-5}} = 1 \times 10^6/\text{mL}$$

Since the diluted suspension represents one-fifth of the original suspension, if the standard dilution of 0.5 mL in 2 mL was used, the number of sperm per mL of the original suspension =
$5 \times (1 \times 10^6) = 5 \times 10^6$.

Volume of the original suspension = 10 mL.

Total sperm in the original suspension = $10 \times (5 \times 10^6) = 5 \times 10^7$/suspension = number sperm/cauda.

If cauda weight = 0.1500 g, total sperm in 0.1500 g of caudal tissue = 5×10^7.

Total sperm per "g" of caudal tissue =

$\frac{5 \times 10^7}{0.1500} = 3.33 \times 10^8$ sperm/g cauda.

This value can also be estimated by the following formula:

$$\frac{\text{Mean No. of sperm in 5 secondary squares}}{\text{caudal weight in g}} \times 50{,}000 \times 50 \text{ (dilution factor)}$$

Record the number of sperm/g cauda to one decimal place.

5. Summary Of Manual Procedure for Sperm Evaluation in Mice and Data Sheet

a. Place instruments and glassware in oven at 37°C. (TT See Note at bottom of page)

b. Put modified Tyrode's in water bath at 37°C. (TT)

c. pH of Tyrode's should be 7.2. (TT)

d. Weigh animal (NT), record weight (NT), bleed animal (TT), euthanize animal (NT), and collect blood again when required (TT).

e. Excise left testis and epididymis. Remove intact epididymis, weigh and record to nearest 0.0001 g. (NT)

f. Cut off left cauda, weigh to 0.0001 g and record. (NT)

g. Place , < 100 uL warm modified Tyrode's on each of 2 slides. Place sperm the size of half a pinhead on the slides and gently mix with Tyrode's, cover-slip and measure motility (40X). (TT)

h. Weigh left testis to 0.0001 g and record. (NT)

i. Place remaining cauda in petri dish with 2 mL PBS, gently chop, swirl, cover, and incubate for 15 minutes at 37°C. (TT)

j. Remove petri dish, ensure homogeneity, and transfer 0.5 mL sample to test tube containing 2 ml PBS. (TT) Transfer 2 aliquots of residual suspension into two 1.5 ml vials (filled to approximately 80%) and place on dry ice for shipment to NTP Archives.

k. Kill sperm by heat, cold, or aldehyde fixatives. (TT)

l. Pipette 5 times and place sample on hemacytometer for count (20X), Repipette and place a second sample on another hemacytometer for a second count. (TT) Note: Count sperm in 5 secondary squares and if less than 5 per square, then count sperm in 2 tertiary squares and record change.

Note: NT = Necropsy Technician TT = Toxicology Technician

DATA SHEET - MOUSE SPERM STUDIES

DATE:_____

LABORATORY _____LAB #:_____

TEST ARTICLE:_____CAS #_____

C # _____ TREATMENT GROUP: _____ANIMAL #:_____

ANIMAL WEIGHT:_____ g * EPIDIDYMAL WEIGHT (LEFT):_____ g *

CAUDA WEIGHT (LEFT):_____ g * TESTICULAR WEIGHT (LEFT):_____ g *

* Enter body weights to 1 decimal place & tissue weights to 4 decimal places

o AUTOMATED MEASUREMENTS

SPERM MOTILITY

field	1	2	3	4	5
motile					
non-motile					

Total Motile:____

SIGNATURE:_____ Total Non-motile:____
Observer A Date

field	6	7	8	9	10
motile					
non-motile					

% Motility (1) :_____

SIGNATURE: _____
Observer B Date

Sperm Count

(2) Vol. Saline (cauda in 2 mL PBS) _____mL

Count1	Count2	Mean

(2) Vol. Caudal Susp. (0.5 mL in 2 mL) _____mL

(3) Sperm Concentration: _____ X 10^6
Per g caudal tissue

SIGNATURE: _____
Observer Date

(1) Total motile/total motile + Non-motile X 100%

(2) Leave blank unless other volume is used. Do not dilute if the number of sperm per secondary square is less than 15.

(3) X(50000) Y (to the nearest 0.1 X 10^6) (X = # sperm counted; Y = dilution factor, which is 10 cc/caudal weight in grams)

NOTE: DILUTION FACTOR WILL CHANGE IF DIFFERENT VOLUMES OF DILUENT ARE USED.

6. Summary Of Manual Procedure for Sperm Evaluation in Rats and Data Sheet

a. Place instruments and glassware in oven at 37°C. (TT See Note at bottom of page)

b. Put test yolk buffer in water bath at 37°C. (TT)

c. Weigh animal (NT), record weight (NT), bleed animal (TT), euthanize animal (NT), and collect blood again when required (TT).

d. Excise left testis and epididymis. Remove intact epididymis, weigh and record to nearest 0.0001 g. (NT).

e. Cut off left cauda, weigh to 0.0001 g and record. (NT).

f. Place < 100 uL of warm test yolk buffer on each of 2 slides. Place sperm sample the size of a pinhead on the slides and gently mix with yolk, cover-slip, and measure motility (40X). (TT).

g. Note: There should be 3X as many motile compared to nonmotile sperm.

h. Weigh left testis to 0.0001 g and record. (NT).

i. Place remaining cauda in petri dish with 10 mL PBS, gently chop, swirl, cover, and incubate for 15 minutes at 37°C. (TT).

j. Remove suspension from oven and pipette 20 times and remove connective tissue. (TT).

k. Place 0.5 mL sample in test tube containing 2 mL PBS. (TT) Transfer 2 aliquots of residual suspension into two 1.5 ml vials (filled to approximately 80%) and place on dry ice for shipment to NTP Archives.

l. Kill sperm by heat, cold, or aldehyde fixatives. (TT).

m. Pipette 5 times and place sample on hemacytometer for count (20X). Re-pipette and place a second sample on another hemacytometer for a second count. (TT).

n. Note: Count sperm in 5 secondary squares and if less than 5 per square, then count sperm in 2 tertiary squares and record change.

Note: NT = Necropsy Technician TT = Toxicology Technician

DATA SHEET - RAT SPERM STUDIES

DATE:_____

LABORATORY _____LAB #:_____

TEST ARTICLE:_____CAS #_____

C # _____ TREATMENT GROUP: _____ANIMAL #:_____

ANIMAL WEIGHT:_____ g * EPIDIDYMAL WEIGHT (LEFT):_____ g *

CAUDA WEIGHT (LEFT):_____ g * TESTICULAR WEIGHT (LEFT):_____ g *

* Enter body weights to 1 decimal place & tissue weights to 4 decimal places

o AUTOMATED MEASUREMENTS

SPERM MOTILITY

field	1	2	3	4	5
motile					
non-motile					

Total Motile:____

SIGNATURE:_____ Total Nonmotile:____

Observer A Date

field	6	7	8	9	10
motile					
non-motile					

% Motility (1) :_____

SIGNATURE: _____

Observer B Date

Sperm Count

(2) Vol. Saline (cauda in 10 mL PBS) _____mL

Count1	Count2	Mean

(2) Vol. Caudal Susp. (0.5 mL in 2 mL) _____mL

(3) Sperm Concentration: _____ X 10^6

Per g caudal tissue

SIGNATURE: _____

Observer Date

(1) Total motile/total motile + non-motile X 100%

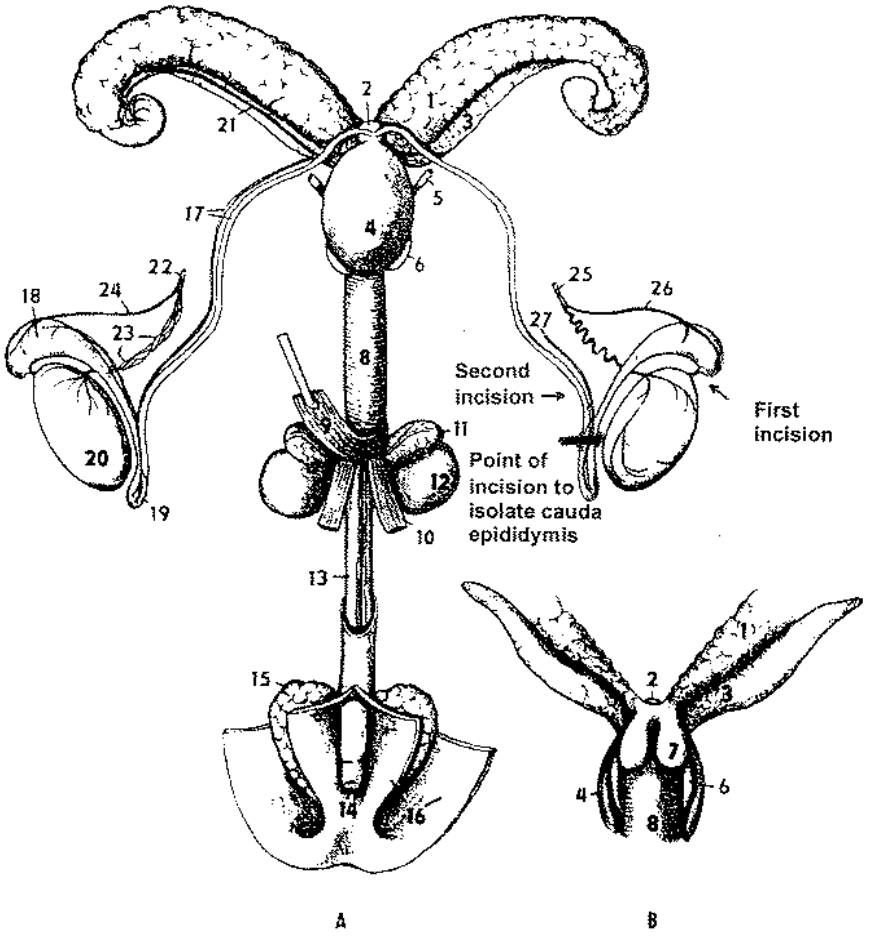
(2) Leave blank unless other volume is used

(3) X(50000) Y (to the nearest 0.1 X 10^6) (X = # sperm counted; Y = dilution factor, which is 50 cc/caudal weight in grams)

NOTE: DILUTION FACTOR WILL CHANGE IF DIFFERENT VOLUMES OF DILUENT ARE USED

F. FIGURES

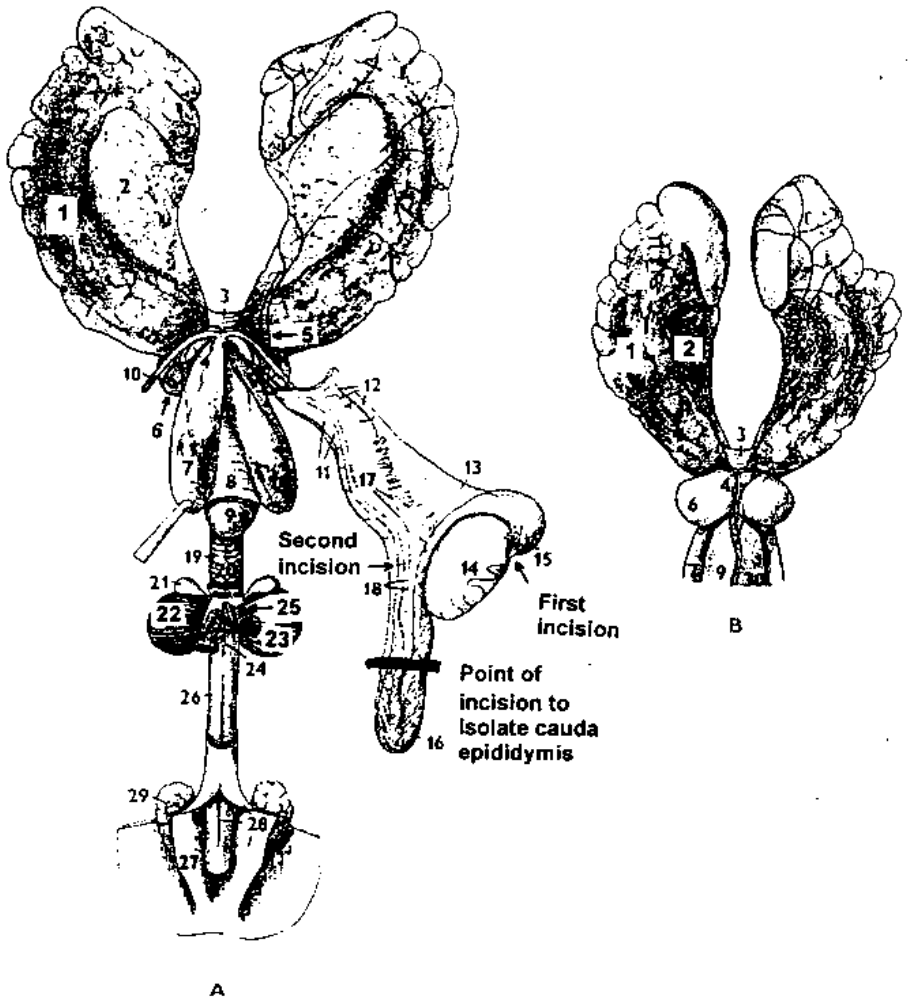
Figure 1. Male **MOUSE** genital organs; view of the ventral (A) and dorsal (B) surfaces.



1 *glandula vesicularis* – vesicular gland
2 *glandula ampullaris* – ampullary gland
3 *glandula coagulationis* – coagulational gland
4 *vesica urinaria* – urinary bladder
5 *ureter* – ureter
6,7 *prostata* – prostate
6 *pars ventralis* – ventral part
7 *pars dorsalis* – dorsal part
8 *pars pelvina urethrae et m. urethralis* – pelvic part of urethra and urethral muscle
9 *m. bulboglandularis* – bulboglandular muscle
10 *m. ischiocavernosus* – ischiocavernous muscle
11 *glandula bulbourethralis* – bulbourethral gland
12 *diverticulum glandulae bulbourethralis* – diverticulum of bulbourethral gland
13 *penis* – penis
14 *glans penis* – glans penis
15 *glandula preputialis* – preputial gland
16 *preputium* – prepuce
17 *ductus deferens v., ductus referentis* – deferent duct, vein of deferent duct
18,19 *epididymis* - epididymis
18 *caput epididymis* – head of epididymis
19 *cauda epididymis* – tail of epididymis
20 *testis* – testis
21 *v. glandulae vesicularis* – vein of vesicular gland
22 *v. testicularis dextra* – right testicular vein
23 *plexus pampiniformis* – pampinifrom plexus
24 *ramus epididymis v. testicularis* – epididymis branch of testicular vein
25 *a. testicularis sinistra* – left testicular artery
26 *ramus epididymidis cranialis a. testicularis* – cranial epididymis branch of testicular artery
27 *a. ductus deferentis* – artery of deferent duct

Popesko, P., Rajtova, V., Horak, J. (1992). *Colour Atlas of the Anatomy of Small Laboratory Animals. Volume 2. Rat, Mouse, Golden Hamster. English translation.* Wolfe Publishing Ltd. ISBN 0 7234 1823 3.

Figure 2. Male **RAT** genital organs *in toto*, showing the ventral surface (A); dorsal view of genital glands, urinary bladder, and dorsal prostate (B).



1 *glandula vesicularis* – vesicular gland
2 *glandula coagulationis* – gland for coagulation
3 *lig. intervesiculare* – intervesicular ligament
4 *glandula ductus deferentis* – gland of deferent duct
5 *rami glandulae vesicularis (a. et v.)* - branches of vesicular gland (artery and vein)
6 *prostata dorsalis* – dorsal prostate
7 *prostata ventralis* – ventral prostate
8 *lig. interprostaticum* – interprostatic ligament
9 *vesica urinaria* – urinary bladder
10 *ductus deferens* – deferent duct
11 *a. et v. ductus deferentis* – artery and vein of deferent duct
12 *a. et v. testicularis, mesorchium proximale* – testicular artery and vein, proximal mesorchium
13 *mesorchium distale* – distal mesorchium
14 *testis* – testis
15,16 *epididymis* - epididymis
15 *caput* – head
16 *cauda* – tail
17 *ramus epididymalis (a.et v.)* – epididymal branch (artery and vein)
18 *ramus ductus deferentis (a. et v.)* – branch for deferent duct (artery and vein)
19 *m. urethralis* – urethral muscle
20 *m. ischiocavernosus* – ischiocavernous muscle
21 *glandula bulbourethralis* – bulbourethral gland
22 *m. bulboglandularis* – bulboglandular muscle
23 *bulbus urethrae (in projectione)* – urethral bulb (in projection)
24 *urethra masculina (in projectione)* – masculine urethra (in projection)
25 *m. bulbospongiosus* – bulbospongious muscle
26 *corpus penis* – body of penis
27 *glans penis* – glans penis
28 *v. dorsalis penis, preputium* – dorsal vein of penis, prepuce
29 *glandula preputialis* – preputial gland
30 *a. et v. vesicalis* – vesical artery and vein

Popesko, P., Rajtova, V., Horak, J. (1992). *Colour Atlas of the Anatomy of Small Laboratory Animals. Volume 2. Rat, Mouse, Golden Hamster. English translation.* Wolfe Publishing Ltd. ISBN 0 7234 1823 3.

Figure 3. Diagram of a rat epididymis showing the site for removal of sperm samples from proximal cauda.

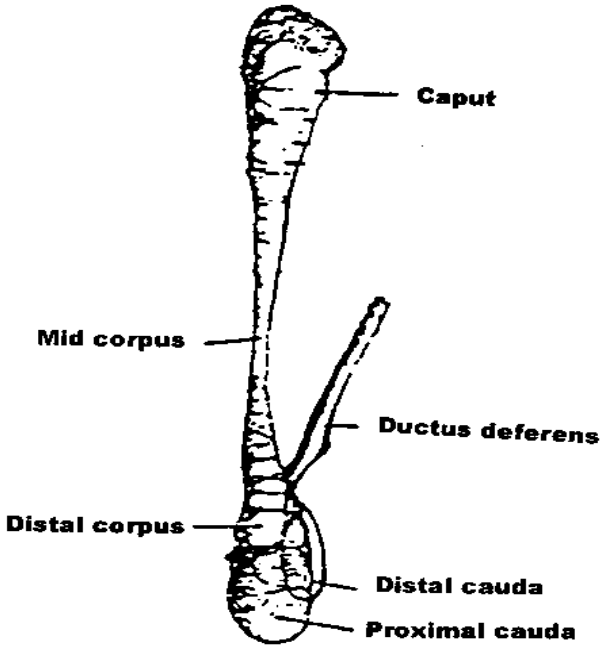
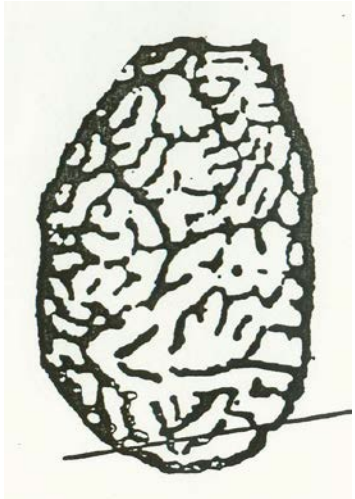
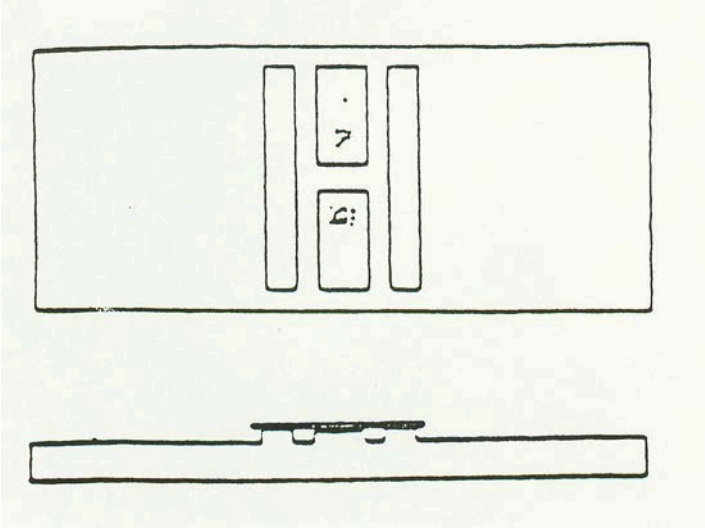


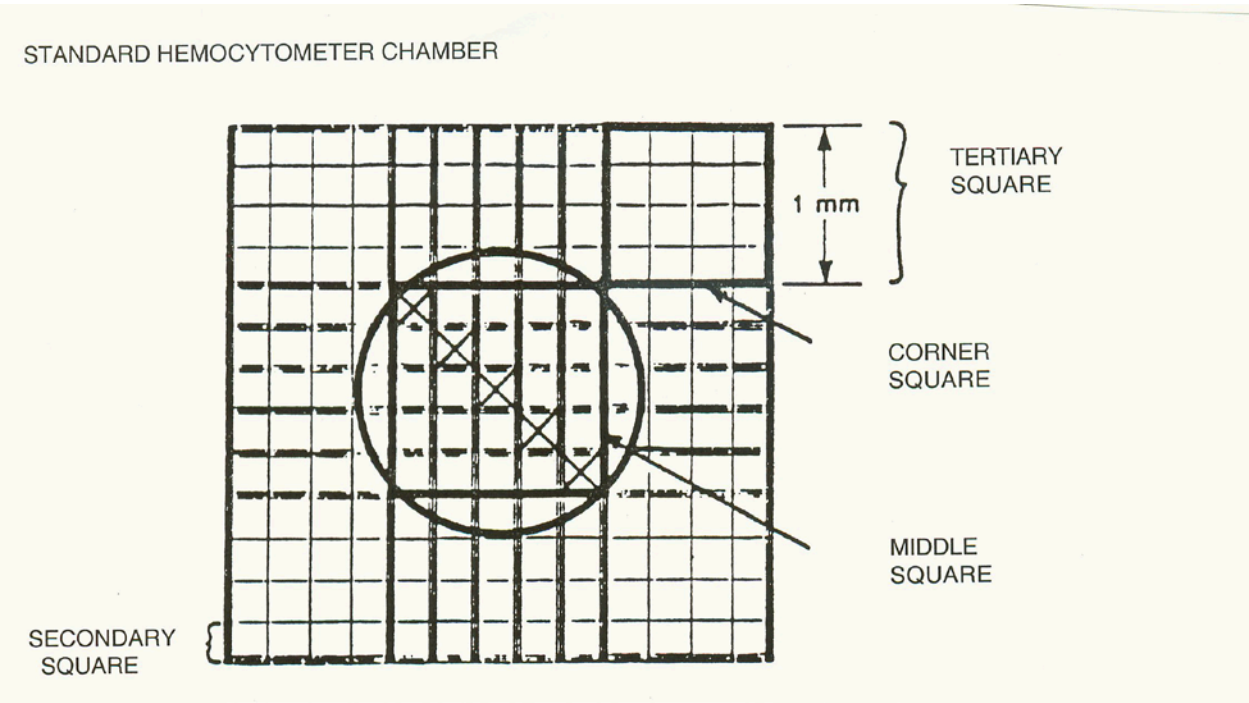
Figure 4: Representation of proximal cauda in mouse.



Recommended portion of cauda adequate for sperm motility evaluations.

Figure 5. Views of hemocytometer.





Volume Occupied by a Primary Square = 2.5×10^{-7} mL

Volume Occupied by a Secondary Square = 4×10^{-6} mL

Volume Occupied by a Tertiary Square = 1×10^{-4} mL

Since sperm count is made in `5` secondary square, it is equivalent to = $5 \times 4 \times 10^{-6}$ mL or 2×10^{-5} mL.

II. VAGINAL CYTOLOGY ASSAY IN MICE AND RATS

A. THE ESTROUS CYCLE

This section provides a quick overview of the estrous cycle and describes the technical procedures for the vaginal cytology assay. Also provided is a list of materials needed.

The existence of a typical estrous cycle in the guinea pig and the associated changes in the vaginal cytology were first reported by Stockard and Papanicolaou in 1917. Shortly thereafter, similar phenomena were reported in the rat and mouse. Vaginal smears obtained from these animals were found to correlate well with changes occurring in the reproductive tract and with the secretion of the ovarian hormones. The most common types of cells present in a vaginal smear are:

- Polymorphonuclear leucocytes
- Nucleated epithelial cells
- Large squamous epithelial cells, also called cornified cells (nucleated or non-nucleated).

The estrous cycle of the mouse and rat is completed in four to five days (polyestrous) although the timing of the cycle may be influenced by exteroceptive factors (light, temperature, etc.). The cycle is roughly divisible into four main phases:

1. Estrus

   This is the period of heat and copulation is possible only at this time. This phase lasts from 9 to 15 hours and is characterized by a high rate of running activity. Many mitoses occur in the vaginal mucosa and as new cells accumulate, the superficial layers become squamous and cornified. The latter cells are exfoliated into the vaginal lumen and their presence in vaginal smears is indicative of estrus. During late estrus there are cheesy masses of cornified cells with degenerate nuclei present in the vaginal lumen; but few, if any, leucocytes are found during estrus. Much of the luminal fluid in the uteri is lost before ovulation.

2. Metestrus

   This occurs shortly after ovulation and is intermediate between estrus and diestrus. This phase lasts for 10 to 24 hours and mating is usually not permitted. Many leucocytes appear in the vaginal lumen along with a few cornified cells.

3. Diestrus

   This phase lasts for 60 to 70 hours, during which functional regression of the corpora lutea occurs. The vaginal mucosa is thin, and leucocytes migrate through it, giving a vaginal smear consisting almost entirely of these cells.

4. Proestrus

   This heralds the next heat and is characterized by functional involution of the corpora lutea and preovulatory swelling of the follicles. Fluid collects in the uteri and they become highly contractile. The vaginal smear is dominated by nucleated epithelial cells that occur singly or in sheets.

B. SUGGESTED LIST OF MATERIALS FOR VAGINAL CYTOLOGY ASSAYS
(To be supplied by the Toxicology Testing Laboratory)

Medicine droppers
Pre-cleaned clear microscope slides (3 X 1") with frosted end (Dakin)
Cover-slips (24 X 60 mm)
95, 80, 70, 50, and 20% ethanol. These grades of alcohol can be prepared from 95% ethanol stock.
Spray Cyte II (Clay Adams 7180) or other fixative
Permount mounting medium and xylene
100-slot slide boxes
0.9% saline solution or phosphate buffered saline
Microscope slide labels
Bibulous paper
Toluidine Blue O (can be purchased from J.T. Baker Chemical Co., Catalog No. W144; if another source is utilized, ensure that the stain is certified for use in histology).
Beakers
Trays
1 N HCL
1N NaOH
Parafilm
Solvent resistant permanent marker

Preparation of Toluidine Blue Stain

2.5 g Toluidine Blue
500 mL 20% ethanol

Mix and allow to sit for one hour before filtering through Whatman Grade filter paper.

C. PROCEDURE FOR OBTAINING A VAGINAL SMEAR FROM MICE AND RATS

1. Two vaginal smears should be prepared from each animal between 8-10 a.m. for the sixteen consecutive last days of the 90-day study or as specified in the study protocol. Dose levels at which these are prepared shall be designated by the NTP project officer for the Toxicology Testing Laboratory.

2. Clear slides with frosted ends are recommended to make vaginal smears (Dakin-Erie 2958). All smears are made on the clear portion of the slide. Slides specifically manufactured for vaginal cytology with 8 sections may also be used with COTR approval.

3. Microscope slides are marked with a solvent-resistant permanent marker into a grid consisting of six squares per slide on the clear portion of the side of the slide with the frosted end. The frosted end is marked with a permanent marker or a pencil to indicate the species/animal#/slide (i.e., 01-M-001B is sufficient at this stage since a label with complete information shall be applied later). The squares are labeled 1 through 6, 7 through 12, and 13 through 16 in the upper right hand corner. This is convenient as it allows the incorporation of six days of smears on one slide.

4. A 3-4" medicine dropper is moistened by aspirating 0.9% saline or PBS solution. A very small amount of saline (up to the shoulder of the medicine dropper, approximately 0.2 ml) is left in the medicine dropper then placed in the vagina (depending upon the strain this depth may be ~ ½ to ¼ inch for rats and less for mice) and the vaginal fluids aspirated back and forth several times.

5. The contents of the medicine dropper are transferred onto the slide within the appropriate square for that animal-day. The use of excessive saline should be avoided to prevent the flow onto surrounding squares. The slide shall be checked for sufficient cell numbers (~10-20 per 100x field) using a microscope. The slides are to be stored in slide folders in a dust-free atmosphere between collection dates.

6. Twenty-four hours after the 16th smear is taken the smears are fixed by applying fixative according to manufacturer's instructions. The fixative shall be held ~8 inches above the slides and sprayed using a back and forth motion. This is done in a fume hood.

7. After the smears are fixed, the slides are allowed to dry completely over night. The slides can be stained the next day or within the next few days.

8. Slides are then loaded in glass racks and dipped in the following solutions:

   a) 95% Ethanol - 30 minutes
   b) 80% Ethanol - 1 minute with gentle shaking
   c) 70% Ethanol - 1 minute with gentle shaking
   d) 50% Ethanol - 1 minute with gentle shaking
   e) 20% Ethanol - 1 minute with gentle shaking
   f) 0.5% Toluidine Blue in 20% Ethanol - 30 – 45 seconds. Personal judgment should be used because Toluidine Blue improves with age.
   g) Running tap water rinse - until tap water runs clear (at this point if slides aren't dark enough when checked under a microscope, dip in 20% Ethanol and put back in the stain; the cornified cells should be stained blue with a purple nucleus visible).

9. The stained slides are blotted gently under bibulous paper. Throw away wet papers because material on a slide can be transferred to other slides. The slides are allowed to

dry overnight and then cover-slipped using Permount or suitable mounting medium. If slides cannot be cover-slipped the following day, store in a slide folder and cover-slip as soon as possible.

10. Slide Labeling and Coding

Each laboratory participating in this project will be assigned an identification number by the NTP-designated SCVCE laboratory. (Contact the SCVCE laboratory to find out your assigned identification number.) Each slide shall contain the following information:
(See example of slides and decoding form provided below).
Line 1: C # (this is the NTP study number).
Line 2: Lab Code - Species - Animal Number & Slide: Example 01-M-001A
01 designates the lab code; M indicates mouse (R indicates rat);
001A designates the randomly assigned number to an animal plus
A indicates first of three slides for that animal (B = second slide, C = third slide)
Line 3: The date the first smear was made
Line 4: The date the last smear was made

C62408 01-M-001A 04-06-06 04-21-06	1	2	3
	4	5	6

C62408 01-M- 001B 04-06-06 04-21-06	7	8	9
	10	11	12
C62408 01-M-001C 04-06-06 04-21-06	13	14	15
	16		

Example: Preparation of slides for observation of the estrous cycle by means of vaginal changes.

11. All decoding information is to remain with the individual testing laboratory and a copy sent to the SCVCE laboratory to be opened only after completing all evaluations and in the presence of the Quality Assurance Officer who will date and sign when opened. Decoding information to be provided is shown below. All slides will be shipped according to instructions provided in Section D below.

LABORATORY: XYZ LAB # 01
TEST ARTICLE: Emodin ROUTE/VEHICLE: Dosed Feed/NTP2000
CAS #: 518-82-1 C #: C62408 STRAIN: B6C3F1/N Mice
DATES SMEARS TAKEN: 4/6/06 - 4/21/06 TSAC DATE: 4/22/06

SLIDE CODE	TREATMENT GROUP	ANIMAL NUMBER	BODY WEIGHT @ TSAC (g)
01-M-001	8000 ppm	113	29.2
01-M-002	Control	61	30.6
01-M-003	1000 ppm	85	29.7
01-M-004	2000 ppm	97	28.9
Etc…….			

D. INSTRUCTIONS FOR SHIPPING SLIDES TO THE SCVCE LABORATORY

Shipping and packaging cost shall be incurred by the NTP testing laboratory conducting the toxicity studies.

Stained and cover-slipped slides shall be randomized by animal and shipped to the NTP SCVCE laboratory within 2 weeks of necropsy. When randomizing by animal number, keep all three slides from each animal together.

A copy of the code for all slides shall be placed in a sealed envelope and sent to the SCVCE laboratory along with the coded slides.

All vaginal cytology slides shall be placed in plastic slide boxes, keeping slides from mice and rats separate.

These slide boxes shall be placed in 350 lb. test cardboard boxes, separated by abundant packaging material for shipment.

An appropriate listing of slides shall be packed in the slide boxes.

Slide boxes shall be packed to avoid any breakage.

Shipping cartons shall be sealed and bound with filament tape prior to shipment.

Slides sent separately in slide set shall be counted as present in the inventory; a copy of the slide set inventory will accompany the major inventory document.

Each plastic shipping box shall be marked with the name and address of the laboratory, the test article, CAS #, C #, Strain, and the range of animal numbers included in that box.

Shipping cartons containing slides and the appropriate information shall be directly mailed to the NTP-designated SCVCE laboratory.

E. SUGGESTIONS FOR IMPROVING VAGINAL CYTOLOGY QUALITY

General instructions to make vaginal smears are described in detail above. A few simple precautions, however, can significantly increase the quality of the slides. These are discussed in this section.

Under each category there are suggestions and/or possible explanations for correcting the potential problems that may be encountered during the preparation of a vaginal cytology smear.

a. Crystallization

This is a frequent problem since most staining solutions easily crystallize upon drying. 0.5% Toluidine Blue in 20% ethanol solution is optimal for staining vaginal smears. We have not noticed any excessive crystallization with this concentration. Mucous crystallizes easily in the presence of stain. Since the mucous is darkly stained, that make the smears hard to evaluate and, furthermore, the presence of crystals distorts the morphology of the cells. Also, debris on the slide will provide crystallization sites. Smears must be made on clean slides and slides should always be stored in a dust-free atmosphere.

b. Debris

Presence of debris in vaginal smears will interfere during the evaluation. Debris usually comes from dirty slides, tissue fragments, or dust in the air. Therefore, all slides shall be precleaned by wiping them with a clean gauze or Kim Wipe. Most of the tissue fragments can be eliminated by carefully aspirating vaginal fluids. It is also important to place all uncover-slipped smears in a dust-free environment so that dust settling on the slides is minimized. Cover-slips, if dusty, shall be cleaned prior to use.

c. Staining

The major problem encountered in this category is the non-uniformity of the stain. This results from the different densities of cells in each smear. By scanning the slide, it is possible to determine if all the smears are adequately stained. If they are not, place the slides back in the alcohol series and then in Toluidine Blue to re-stain them. Be careful not to stain cells too dark because it severely interferes with evaluation.

d. Clumping and Cell Density

The clumping of cells is mainly due to an excess number of cells on the slide. This can be remedied by not placing too many cells on the slide. If the smear is too dense, it will stain heavily and clump. If too much saline is used in making the smear, the cell density of the smear will be very low. This causes the smear to have too few cells to allow accurate evaluation. It must be added that some of the stages of the estrous cycle have a higher density of cells than others. This adds to the problem of cell density.

e. Air Bubbles

Air bubbles are a major problem especially when they occupy a large portion of the slide. They are mainly a technical problem caused by faulty technique and/or carelessness. More mounting medium may be needed to cover the entire slide or better pressing of the cover-slip to squeeze out air bubbles is required. The use of Flo-Tex or similar medium may also alleviate this problem.

F. VAGINAL CYTOLOGY QUALITY CODES

(Codes used by SCVCE Laboratory for the Evaluation of Slides)

Code		Subcode[1]	
00	Excellent	- 1	Refers to Day 1 only
01	Good	- 2	Refers to Day 2 only
02	Moderate crystallization	- 3	Refers to Day 3 only
03	Heavy crystallization	- 4	Refers to Day 4 only
04	Moderate debris	- 5	Refers to Day 5 only
05	Heavy debris	- 6	Refers to Day 6 only
06	Debris surrounding cells	- 7	Refers to Day 7 only
07	Staining too light	- 8	Refers to Day 8 only
08	Cornified cells stained lightly	- 9	Refers to Day 9 only
09	Staining too dark	-10	Refers to Day 10 only
10	Non-uniform staining	-11	Refers to Day 11 only
11	Cells heavily clumped	-12	Refers to Day 12 only
12	Few cells	-13	Refers to Day 13 only
13	No cells	-14	Refers to Day 14 only
14	Air bubbles (>5% < 20% slide)	-15	Refers to Day 15 only
15	Air bubbles (>20% slide)	-16	Refers to Day 16 only
16	**Cells incorrectly applied to the slide (i.e., applied on the side of the slide lacking the frosted end or not in the appropriate grid on the clear portion of the side of the slide with the frosted end).**		

[1]Subcodes are used in conjunction with code when that remark applies to a particular day (e.g., 13-2 means no cells on Day 2).

BLANK PAGE

APPENDIX 6 - Pathology

Section A - Procedures for Collecting Non-Reproductive Organs

In all studies, the necropsy procedure includes external examination of the animal including all body orifices and gross examination of all organs and tissues. All organs and tissues with gross lesions are to be collected. Following is a list of organs/ tissues that may need to be collected during necropsy and for which the collection procedure is described below. This list is not exhaustive, however, and other organs or tissues may need to be collected if they contain gross lesions or are a known target organ (refer to the specific study protocol for instructions on which organs / tissues are to be collected).

Brain
Clitoral glands
Esophagus
Eyes
Femur
Gallbladder (mouse only)
Gross lesions
Harderian glands
Heart and aorta
Intestine, large (cecum, colon, rectum)
Intestine, small (duodenum, jejunum, ileum)
Lungs and mainstem bronchi
Lymph nodes
- mandibular and mesenteric
Muscle, thigh (if neuromuscular signs are present)
Nerve, sciatic, tibial (rat only), trigeminal (with ganglion)
Nose (nasal cavity and nasal turbinates)
Oral cavity, larynx, and pharynx
Pancreas
Parathyroid glands
Preputial glands
Salivary glands
Skin
Spinal cord
Spleen
Stomach (forestomach and glandular)
Thymus
Tongue
Trachea
Urinary bladder
Zymbal's glands

1. GENERAL INSTRUCTIONS

   A. Organs/tissues shall be examined in situ, then dissected from the carcass in the manner specified below, re-examined, including cut surfaces, and fixed in 10% neutral buffered formalin (NBF). The exception to this is that the eyes are fixed in modified Davidson's solution.

   B. All gross lesions shall be described by the supervising Pathologist and recorded using the terminology in the Pathology Code Table (PCT) (in TDMS or other data collection system as directed by the COTR) inclusive of morphologic lesion; anatomic site; quantity; size or volume in

millimeters or milliliters; number; shape; color; and consistency. Each gross observation shall be correlated with a microscopic evaluation.

C. All protocol-required organs (with the possible exception of skin, mammary glands, bone and muscle) shall be saved and fixed in their entirety, e.g. no part of the organ shall be discarded.

D. Tissues saved for histopathology shall be fixed at a thickness not to exceed 0.5 cm except as stated below.

E. Tails or other body parts that have been used in any way for animal identification during the in-life phase of the studies shall be saved in formalin with the animal tissues. If ear tags or other identifying methods are used for any reason, these too shall be saved in formalin with the animal tissues.

- Eosin shall be added to the stock formaldehyde solution in sufficient quantity to impart a pink tinge to the NBF.
- Multiple representative portions of large or heterogeneous tissue masses including surrounding unaffected tissues must be fixed. Masses less than 0.5 cm diameter may be fixed in their entirety.
- The number of grossly visible nodules/masses in an organ shall be recorded up to five per organ; after the five largest nodules/masses have been counted and sampled, the IANR shall show under “Notes” the number as "greater than five" for that organ.
- Organs typically weighed in a sub-chronic (“90-day”) toxicity evaluation are: liver, thymus, right and left kidney, heart, and lungs. These organs shall be weighed to the nearest 10 mg except for thymus, which shall be weighed to the nearest 1.0 mg. Organ/body weight ratios shall be calculated.

2. SPECIFIC INSTRUCTIONS FOR ORGAN / TISSUE COLLECTION

a. *Alimentary Tract:* The oropharynx, esophagus, stomach, small and large intestines, and rectum shall be opened only as described below:

i. Oral cavity, pharynx, and larynx shall be carefully examined grossly. If any abnormalities, including tumors and/or lesions, are noted, the tissues shall be examined microscopically.

ii. Remove mandible to allow visualization of the tongue and posterior pharynx.

iii. If there are lesions present in the tongue, take a cross-section through the entire tongue that includes the lesion(s) and adjacent tissue.

iv. Take a routine cross section of esophagus with the trachea and esophagus in one section. Open remaining esophagus (but not the stomach) and examine.

v. Split the pelvis and remove the entire gastrointestinal tract including the anus.

vi. Separate the stomach and duodenum by transecting at the junction of the pylorus and duodenum. Inject the stomach with NBF (mouse = 2 ml; rat = 5 ml) and immerse in NBF. The stomach shall be opened and examined at trimming.

vii. Take cross sections consistently from the same area for each tissue: duodenum (to be taken exactly 1 cm from the pylorus), jejunum, ileum, cecum, colon, and rectum. Include Peyer's patches with these sections where possible.

viii. Open and examine the rest of the intestinal tract. Areas with lesions shall be individually identified and pinned flat on paper or cardboard or placed in a cassette for fixation, labeled as to location, and recorded on the IANR.

ix. For dose range finding studies the entire intestine does not need to be opened, however sections of suspect lesions shall be taken.

b. *Pancreas*: Collect a section of pancreas at least 1 cm^2 for rats or 0.5 cm^2 for mice and immerse in 10% NBF.

c. *Lungs*: Remove the lungs with the remaining portion of the trachea attached. Externally examine and weigh these tissues. After weighing, the trachea and lungs shall be infused by introducing NBF (approximately 1-2 ml for mice and 4-8 ml for rats) into the trachea until the lungs are completely filled to **normal inspiratory volume**. Then the trachea shall be tied, proximally, and the organs immersed in NBF.

d. *Heart*: The heart shall be removed at the base with cranial aorta and be free of pericardial sac. The heart shall be gently squeezed to remove blood from chambers. The heart with attached aorta shall be externally examined and fixed in NBF in its entirety. If blood has clotted, chambers shall be opened and clots removed before weighing.

e. *Thymus*: The thymus shall be carefully dissected from adjacent tissues, weighed, and placed in a labeled cassette to avoid being lost.

f. *Lymph Nodes*: Dissect all lymph nodes free and placed in a labeled cassette to avoid being lost.

g. *Parathyroid Glands*: The parathyroid glands should be included in the section of thyroid gland.

h. *Spleen*: Remove the spleen and immerse in 10% NBF.

i. *Urinary Bladder*: Collect and fix urinary bladder in a manner that will minimize twisting and curling. Remove adipose tissue surrounding the bladder when collecting. Distended urinary bladders shall be fixed as is. Contracted, empty bladders shall be partially distended with formalin. Care shall be taken to insert the needle into the lumen of the bladder. Insertion of the needle into the bladder wall may result in severe artifact. Urinary bladders shall be opened and examined after fixation at trimming.

j. *Salivary Glands*: The submandibular, parotid, and major sublingual salivary glands are closely associated with the mandibular lymph node in the ventral cervical region. These may be removed en masse and immersed in 10% NBF as a single section.

k. *Eyes*: The eyes (in modified Davidson's fixative) shall be dissected free and placed in a labeled cassette to avoid being lost.

l. *Harderian Glands*: The Harderian glands shall be dissected free and placed in a labeled cassette to avoid being lost.

m. *Zymbal's Glands*: Collect the Zymbal's glands and immerse in 10% NBF. If necessary, the Zymbal's glands may be placed in a labeled cassette prior to fixation to prevent loss.

n. *Nose (Head)*: The turbinates and tissues of the nasal cavity shall be fixed by gently inserting a blunt needle attached to a syringe into the nasopharyngeal duct and instilling NBF in the nose until drops appear at the external nares. After fixation and removal of the pituitary, the head is to be decalcified and sections of the nasal cavity taken as specified in Appendix 6.

o. *Spinal Cord*: Expose the spinal cord by removing some of the dorsal processes of the vertebral bodies over the cervical, mid-thoracic, and mid-lumbar regions prior to immersion fixation of the entire cord within the vertebral canal. If specified by the COTR the following may be required: three sections of vertebral column with spinal cord in situ (cervical, mid-thoracic, and lumbar) shall be removed and placed in NBF. Following NBF fixation the vertebral sections shall be decalcified prior to trimming. Fixation and trimming of the cord must be accomplished to provide optimal quality sections of anterior (first/second) cervical, mid-thoracic, and mid-lumbar (lumbar intumescence of spinal cord) segments. Avoid excess handling to minimize histological artifacts.

p. *Preputial/Clitoral Glands*: Remove the preputial or clitoral glands and immerse in 10% NBF. If necessary, the glands may be placed in a labeled cassette prior to fixation to prevent loss.

q. *Skin*: A section of skin, at least 1.5 cm square, with subcutaneous tissue attached, shall be collected from the inguinal region. Prior to immersion in 10% NBF, the skin shall be placed, subcutaneous side down, on a piece of cardboard or index card to prevent curling during fixation.

r. *Skeletal Muscle*: A section of skeletal muscle from the thigh (hamstring or quadriceps muscle), at least 1 cm X 0.5 cm X 0.5 cm, shall be collected and immersed in 10% NBF.

s. *Peripheral Nerves*: Remove only sufficient muscles surrounding the right and left sciatic (rat and mouse) and right and left tibial (rat only) nerves (Figure 1) to expose them, leaving the nerves and muscles attached in the carcass and then fixed in NBF with the carcass. The right and left leg shall be submitted to the NTP Archives with the remaining wet tissues unless the individual SOW requires histopathology of the tibial and sciatic nerves or there were neurologic signs observed during the study then the right sciatic and right tibial nerves shall be trimmed for histopathology.

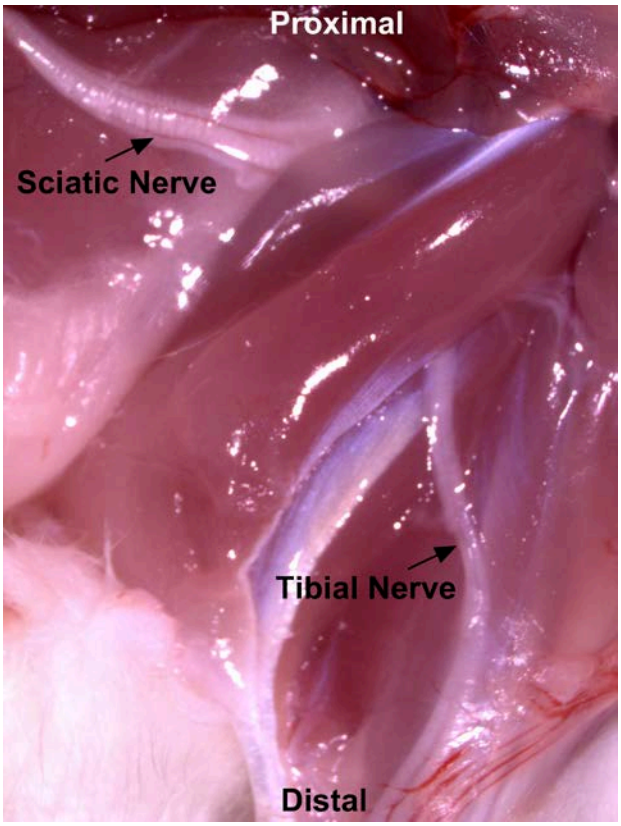


Figure 1: Muscles of the hind limb dissected out from lateral surface to demonstrate the sciatic and tibial nerves.

Brain: The calvarium shall be removed for examination of the brain and pituitary gland. The brain (with olfactory bulbs) shall be removed for fixation in NBF. The pituitary gland and both trigeminal nerves and ganglia shall be left in the calvarium for fixation to avoid excess handling and minimize histological artifacts (Figure 2). Brain removal shall include acquiring the olfactory bulbs in a manner that minimizes damage to the caudal nasal cavity. The nasal bones shall not be removed. The head is to be retained for fixation and then decalcification in order to obtain sections of the nasal cavity taken as specified in the Trimming Section.

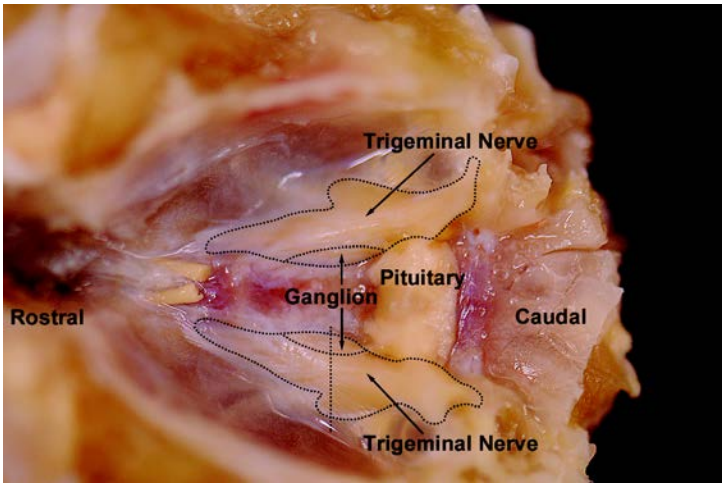


Figure 2: Trigeminal nerve and ganglion.
Vertical dotted line approximates the location of each.

t. *Bone (Femur)*: At the end of the necropsy, one of the femurs shall be disarticulated at both ends, stripped of excess tissue, and immersed in 10% NBF.

Section B - Methods for Trimming Non-Reproductive Organs and Tissues

Specific methods for trimming non-reproductive tissues are as follows:

1. Parenchymal organs shall be free of adjacent tissues and trimmed to allow the largest cross-sectional surface area possible for microscopic examination.
2. Multiple portions of tumors or masses shall be trimmed and submitted if these are large or variable in appearance. Surrounding, normal tissue shall be included if possible with the tumors. Careful documentation of the number of sections taken per mass shall be maintained on the IANR so multiplicity can be determined.
3. For the lung, an entire coronal (dorsal; perpendicular to the sagittal plane and parallel to the long axis of the body) section of both the right and left lungs, including the mainstem bronchi, shall be submitted. In mice, the entire lung pluck (with heart removed) may be embedded dorsal surface down (such that the dorsal surface will be presented for microtomy). In rats, the left and right lungs may be embedded, dorsal surface down, in separate blocks (they may be trimmed to fit the blocks if necessary). Additionally, one section of each mass lesion (nodule or tumor) shall be prepared up to five for each organ. If there are more than five lesions, the five largest tumors shall be sectioned. Adjacent normal tissue shall be included along with the tumor.
4. If neurologic signs were observed clinically, then evaluation of CNS shall be discussed with the COTR.
5. Hollow organs shall be trimmed and blocked to allow a cross-section slide from mucosa to serosa. Since the stomach is infused with formalin at necropsy, it will be opened and the mucosa examined for gross lesions at trimming. The stomach must be cut first through the midsagittal plane thereby dividing it into two equal halves (see Figure 3). The section taken for histology shall include the entire greater curvature of the stomach to include forestomach, glandular stomach, and pyloric region. The section can be further divided into two or three pieces to allow for convenient placement on the slide. Any gross lesions must also be embedded and sectioned.

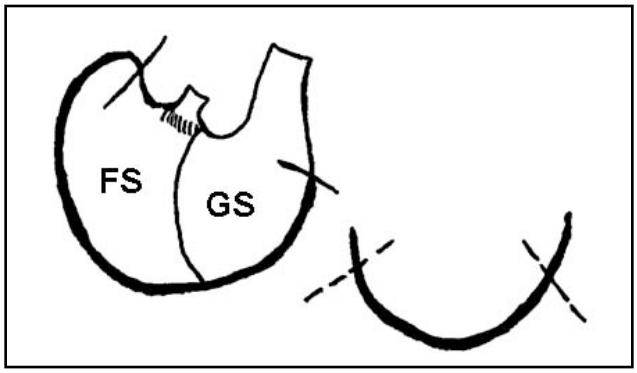


Figure 3: Profile of the stomach through a midsagittal plane on the left, forestomach (FS) and glandular stomach (GS). Shaded area represents location of section. Profile of required section is shown on the right.

6. Following fixation, the trachea shall be opened to the level of the hilus and grossly examined. Any gross lesions in the trachea shall be recorded in the IANR, trimmed, and examined microscopically.
7. After decalcification of the head, three separate sections of the nasal cavity shall be taken at (1) the level of the incisor teeth, (2) midway between incisors and first molar, and (3) middle of second molar (olfactory region). The remainder of the nasal cavity and turbinates shall be carefully

examined for gross lesions at this time. The gross lesions shall be recorded on the IANR. (See Maronpot, Ed. (1999). *Nose, Larynx and Trachea in Pathology of the Mouse*, p. 261. Cache River Press.)

8. Seven cross-sections of the brain taken at levels (brain matrix molds may be used) shown in Figure 4a (rat) and 4b (mouse) shall include: (1) olfactory bulb (mid-level); (2) fronto-parietal cortex including basal ganglia (1-2 mm cranial to the optic chiasma); (3) mid-parietal cortex and thalamus (mid-point of the infundibulum); (4) mid-brain with substantia nigra and red nucleus (mid-point of anterior colliculus); (5) posterior colliculi (mid-point of posterior calliculus); (6) mid-cerebellum including cranial nerve VIII; and (7) posterior medulla through the area postrema (2-3 mm anterior to termination of the cerebellum). If small brains preclude obtaining seven quality sections, a minimum of five sections shall be obtained to include sections 1-4 and 6 corresponding to Figure 5b below. Sections 'b' through 'h' shall be placed in the cassette (large cassette for thick sections) with rostral surface down for sectioning. If any lesions are observed after sectioning, they shall be noted on the IANR.

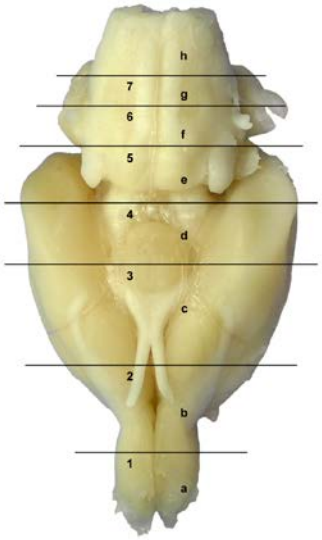


Figure 4a

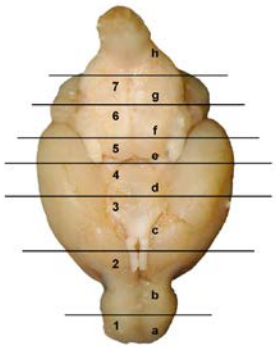


Figure 4b

9. Should the individual SOW require histopathology of the spinal cord or if there were neurologic signs observed during the study then the vertebral column shall be decalcified and trimmed to obtain transverse sections through: (1) anterior (first/second) cervical segment (C1/C2); (2) mid-thoracic segment; and (3) mid-lumbar (at intumescence) segment.

10. The left and right trigeminal nerves and ganglia will be fixed *in situ* and submitted to the NTP Archives with the remaining wet tissues. Should the individual SOW require histopathology of the trigeminal nerve or if there were neurologic signs observed during the study then after fixation the right trigeminal nerve and ganglion shall be dissected from the cranium to obtain a cross-section including the ganglion (Figure 1). Should the individual SOW require histopathology of the tibial and sciatic nerves or if there were neurologic signs observed during the study then the right sciatic (rat and mouse) and right tibial (rat only) nerves shall each be trimmed to obtain one transverse and one longitudinal section (at least 0.7 cm in length). Transverse sections shall include associated skeletal muscle to stabilize the transverse plane and to obtain quality sections of the nerves.

11. The eyes are to be capped off perpendicular to the corneo-scleral junction to permit penetration of chemicals during processing and paraffin infiltration at embedding. The eyes are embedded with the harderian glands and shall be step-sectioned until a section includes both the lens and optic nerve.

12. The pancreas shall be trimmed to allow the largest surface area possible for examination. A portion

of pancreas (about 1 cm^2 for rats and 0.5 cm^2 for mice) shall be embedded flat to provide a section through the frontal plane of the organ rather than a transverse plane.

13. Skin shall be cut from animals so that the orientation of anterior and posterior edges is well defined. For example, the cut edge (dermis) of the anterior edge of the sample can be marked with indelible ink (India) or trimmed in the shape of an arrow. Before placing in formalin, the skin shall be placed with the dermal surface down on a piece of index card to keep the sample flat during fixation.

14. The (sub)mandibular and major sublingual salivary glands are closely associated and together comprise an oval dorsoventrally compressed structure in the ventral cervical region. The mandibular lymph node(s) and parotid gland are located at the cranial border of these salivary glands. The left mandibular and left major sublingual salivary gland and the mandibular lymph node can be embedded as a single unit and must be sectioned through the frontal plane to include the three organs (see figure).

15. A single transverse section of the spleen must be taken at the point of greatest width. If the spleen is diffusely enlarged due to leukemia or lymphoma, the transverse section can be trimmed on one side to allow placement on the slide.

16. A section of the immersion-fixed heart shall be sliced longitudinally from the base through the apex so that all four chambers are visualized (Figure 5). The left ventricle and the aorta shall be used for orientation. The largest, most visible structures of the heart are the ventricles. Note the position of the aorta emerging near the left ventricle out of the base of the heart and making an arching curve back to the right. After the ventricles and the aorta are located, the heart is held with the base and the left ventricle facing upward and toward the prosector. Thus, the base of the heart is seen when the prosector views it from above. The trimming blade (scalpel or razor blade) is placed on the base of the heart so both atria, aorta and both ventricles are included. The cut is then extended straight downward to the apex of the heart. Immediately the cut surface shall be checked for potential presence of thrombi (i.e., thrombus in the left atrium), and if present, the contents should be left in place (not touched during the trimming) as well as evaluation of the valves.

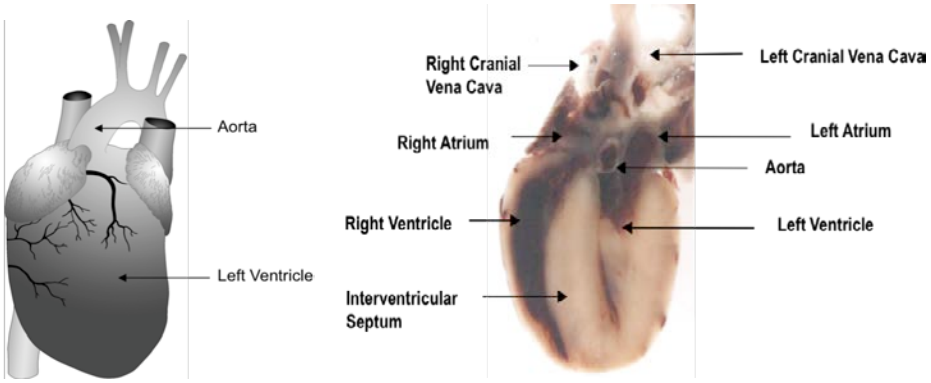


Figure 5. The parts of the heart and great blood vessels demonstrate the plane of section desired.

17. The distal 1.5 cm (minimum length for rat) or 1 cm (minimum length for mouse) of the femur must be sectioned through the frontal plane to include the articular cartilage and articular surface, the femoral condyles with epiphyseal plate, and diaphysis with bone marrow. Sections of bone must include the joint surface and marrow to be considered complete.

Section C - Procedure for Ovarian Follicle Counts

1. PROCEDURE

In some studies, quantitative assessment of the ovarian follicles (ovarian follicle counts) shall be required. The study protocol may indicate the requirement for ovarian follicle counts, but in some cases where ovarian follicle counts are not required by the study protocol, the COTR may request ovarian follicle counts based on the results of the study.

Ovarian follicle counts may be required for one or more generations within a study. Those generations in which follicle counts are to be generated will be identified in the study protocol or the request for ovarian follicle counts.

Ovarian follicle counts will typically be performed on control and high dose groups, but may be required on mid and low dose groups in some cases to determine a NOEL (No Observed Effect Level).

Slides shall be prepared from a minimum of 10 randomly selected animals per treatment group. In some cases, the COTR may request more animals per group be used in ovarian follicular counts.

Follicle counts from both ovaries must be generated for each animal selected. If only one ovary is available, then another animal must be selected. If no other animals are available, then a follicle count from the one available ovary is acceptable.

Ovarian follicle counts will typically be done by manually counting the follicular stages. An automated or semi-automated method for quantitation of ovarian follicles using an image analysis program or another, appropriate, computer program may be used. However, **any automated or semi-automated method must be validated and approved by the NTP COTR prior to use in NTP studies.**

2. SLIDE PREPARATION

A minimum of 5 sections from each ovary (i.e., left and right) from each animal shall be examined. Requirements for preparing the ovarian sections are as follows:

The sections must be at least 100 μm apart.

All sections must be taken from the middle third of the ovary. If this is not possible, then as many sections as possible must be taken from the middle third and the remainder taken from inner most portion of the residual ovary.

All sections must be longitudinal sections (i.e., parallel to the long axis of the ovary).

Sections may be stained with H&E or a specialized staining technique to improve visualization of primordial follicles. Immunohistochemical staining for PCNA may be used, but heat-induced epitope retrieval and high antibody concentrations must be used to ensure staining of all follicular stages (Muskhelishvili, et al., 2005). Other immunohistochemical staining techniques may also be used. However, **all specialized staining and immunostaining techniques must be well-characterized in the scientific literature or validated and approved by the COTR prior to use in NTP studies.**

3. REPORTING

The total number of primordial, growing (primary and secondary; preantral), and antral follicles and corpora lutea shall be recorded for each ovary by animal number.

Any other lesions, not noted in the original ovarian section, must be reported.

Statistical analysis shall be performed on each follicle type and corpora lutea as well as the combination of primordial and growing follicles.

References

Bolon B, Bucci TJ, Warbritton AR, Chen JJ, Mattison DR, Heindel JJ. (1997) Differential follicle counts as a screen for chemically induced ovarian toxicity in mice: results from continuous breeding bioassays. *Fundam Appl Toxicol* 39:1-10.

Muskhelishbili L, Wingard SK, Latendresse JR. (2005) Proliferating cell nuclear antigen – a marker for ovarian follicle counts. *Toxicol Pathol* 33:365-8.

Section D - Tissue Collection Procedures for RNA, DNA and Protein Assays

The following information is supplied for sampling, freezing and storage of tissues for later analysis of RNA, DNA and protein, if required by the study protocol. (Analyses shall be conducted at an NTP-specified laboratory.)

When frozen tissue samples are collected for these types of assays, it is important to recognize that tissue collection speed is critical because the integrity of these samples decreases rapidly following euthanasia. Therefore, all samples shall be collected and processed in a maximum of 5 minutes from euthanasia of an animal. It may be necessary to limit the number of frozen tissue specimens collected from a single animal or study for these types of assays. It may also be necessary to have specific designations for necropsy staff (such as prosector, recorder, frozen tissue processor) to streamline the sample collecting and processing/freezing for each animal/tissue/necropsy station. Conducting laboratories shall configure the necropsy and sample collecting process in a way that optimizes collection of these samples within the 5-minute timeframe if such samples are to be collected in a study.

Tissue samples shall be quickly removed from the animal. Small organs such ovary, adrenal gland, etc may be placed directly into cryotubes; larger organs shall be minced into approximate 2 x 2 mm cubes while kept cold, and frozen as quickly as possible. For example, tissue may be minced in a weigh boat or similar container on top of dry ice (with bottom of weigh boat in full contact with dry ice). After mincing, a small amount of liquid nitrogen may be poured into the weigh boat over the tissue and stirred until the tissue is frozen. Frozen tissue shall be put into RNase-free, DNase-free, pyrogen-free 5 ml screwcap containers (with external threads and lip seal) that have been properly labeled in a permanent manner. Filled containers shall be immediately placed in liquid nitrogen and then transferred to a -120 C freezer for samples that are to be stored for longer than 1 year. For samples to be stored for less than one year, samples may be kept in a -80 C freezer.

In some cases, NTP may request that harvested tissue specimens be immediately submerged in a Tissue Reagent (such as Allprotect or other similar product) for stabilization of RNA, DNA and protein. Use of an agent such as this eliminates the need for dry ice or liquid nitrogen.

NTP will supply test-article- and protocol-specific information as needed for tissue collection, depending on the organs/ tissues collected and anticipated analyses. For example, tissues such as prostate may require other processing steps.

NTP may request that frozen samples be shipped to the NTP Repository. Prior to shipment, the Frozen Tissue Bank Coordinator is to be contacted by phone and by e-mail to establish the time of shipment for frozen samples and associated paperwork. A 24-48 hour notification is required. Cryotubes shall be placed in a 5” x 5” box with a grid in sequential order using the sample number. The outside of the box shall be labeled to show the phase of the study, the treatment, the generation, group numbers, animals numbers and the name of the study laboratory. Samples shall be shipped by overnight delivery on Monday, Tuesday or Wednesday to ensure proper receipt of specimens. Frozen samples are to be shipped on dry ice in boxed Styrofoam containers. A sample inventory list must accompany the shipment. The inventory list must be approved by the COTR prior to shipment.

Section E - Preparation of Mammary Gland Whole Mounts in Mice and Rats

In this procedure, the entire 4th and 5th mammary glands can be removed from rodents as a single tissue. The tissue is slide-mounted, fixed, stained with carmine alum, and made transparent to allow for evaluation of 3-dimensional epithelial structures. The whole mount procedure enables determination of accelerated or delayed mammary gland development.

I. COLLECTION OF THE INTACT MAMMARY GLAND

A. Equipment and Supplies

1. Fine curved scissors
2. Dissection board
3. Straight scissors
4. Holding pins
5. Fine curved forceps
6. Charged slides
7. Curved, serrated forceps
8. 70% ethanol
9. Baking or cafeteria style trays for stacking slides

B. Procedure

1. Euthanize animals as designated in the study protocol.
2. Lay the animal on its back on a dissecting board.
3. Stretch the rear feet into an inverted V and pin feet down (holding pins or small gauge needles work well).
4. Wet the animal's abdomen and rear legs with 70% ethanol (if necessary to reduce hair in the gland preparation).
5. Make a skin incision from the pubis to the rib cage, being careful not to cut through the internal abdominal wall (see Figure 1).
6. Make an incision from the origin of the first incision (at the pubis) along the medial aspect of each rear limb, forming an inverted Y (see Figure 1).
7. Carefully grasp the skin (and not the gland attached to it) with the curved (back) side of a serrated forceps at the center of the inverted Y (i.e., at the origin of the incisions at the pubis).
8. Peel the skin back and pin to the dissection board (or other flat surface such as a cooler top). This exposes the 4th and 5th mammary glands (and 6th in the rat). In the rat, the 5th and 6th glands may need to be separated by cutting through the gland at the leg.

9. Using the back side of the curved forceps (not the tips), gently separate the fat pad containing the 4th and 5th glands (leave the 6th gland in rats) from the skin at the point where the skin is pinned and lift it away from the skin, cutting the attachments to the skin as you go (with fine scissors). Use long, smooth cuts rather than short snips to prevent alteration of the gland morphology and the formation of air bubbles on the slides. The forceps may need to be repositioned further down the fat pad to prevent the gland from tearing.

10. When the entire fat pad (to the back of the animal) with the 4th and 5th mammary glands has been separated from the skin, make a straight cut parallel to the animal's body and detach the fat pad/mammary glands.

11. Spread the mammary tissue on a dry slide, skin side down, with the forceps (completely spread out), one fat pad/mammary gland set (glands 4/5 as a single unit) per slide. The thickest part of the gland should be adjacent to the slide label and the lymph node should be near the center of the slide.

C. Helpful Hints

1. Removing the gland in one piece may be difficult - lift gently without squeezing hard. Be sure to use the back side of the curved forceps and not the pointed ends to work with the tissue. The gland may be rolled onto itself to prevent tearing.

2. Make sure to get the tissue adjacent to the animal's dorsum (back), as that is a glandular area in adult animals.

3. Remove as much of the fat pad as possible, width-wise, to ensure that the nipple region of the 4th gland is included. There will typically be plenty of tissue length-wise. To do this, insert the tip of the scissors adjacent to the skin and under the fat pad, and open them, separating the fat pad from the skin.

II. FIXING AND STAINING PROCEDURE

A. Equipment and Supplies

1. Carnoy's Fixative:
   a. 6 parts 100% ethanol
   b. 3 parts chloroform
   c. 1 part glacial acetic acid

2. Carmine Alum Stain:
   a. 1 gram carmine (Sigma C1022)
   b. 2.5 grams aluminum potassium sulfate (Sigma A7167)
   c. Fill to 500 ml with distilled water
   d. Boil for 20 min.
   e. Adjust final volume to 500 ml with distilled water
   f. Filter (to remove residues) into a dark or foil-wrapped bottle
   g. A small amount (1 crystal) of thymol may be added as a preservative
   h. Refrigerate

   Expiration date is 3-4 months (6 months with thymol) from date of preparation. Carmine can be removed from glassware with 100% ethanol.

3. Glass microscope slides (use 2x3” slides for PND 70-90 rats; the same or standard sized slides may be used for all other animals)

4. Parafilm (2 x 3 in.)

5. Glass slide dishes

6. 70% and 95% ethanol

7. Xylene

8. Permount (Fisher Scientific)

9. Glass disposable pipets

10. Rubber suction bulbs

11. Cover slips (appropriate size for glands removed - will vary with animal age)

12. 50 ml conical tubes filled with water and capped, or another object with a flat surface that weighs 415-420 grams (e.g., a beaker with water)

13. Refrigerator with space for cooling tissue (slide trays may be stacked)

B. **Procedure**

1. Once the mammary tissue has been spread onto slide, press on the tissue with your fingers to remove bubbles that may be under the tissue, place a 2x3 inch rectangle of Parafilm on the gland and cover with another glass slide. Place an inverted 50 ml conical tube filled with water (or another object weighing 415-420 g) on the mammary tissue/glass slide sandwich.

2. Compress the mammary tissue in a refrigerator for 30 minutes to several hours, depending on its thickness (PND 4 or 11 for 20-30 min.; rat PND 45 for 1-2 hours; PND 70-90 rats may require as much as 2-5 hours).

3. Remove the 50 ml conical tube and the top glass slide, and peel the Parafilm back from one end, being careful not to loosen the gland from the slide. Place the slides in a glass staining tray. Fix the tissue in Carnoy’s Fixative (section II.A.1. above) for 4 to 48 hours, depending on thickness, at room temperature. Most mammary tissue from PND 32-45 rats and smaller can be fixed for 18-24 hours (overnight). Tissue from PND 90 rats may require more time. If white areas are present in the mammary tissue after fixing (more opaque than rest of gland), change the fixative and allow those glands to fix for an additional 24 hours.

4. Soak in 70% ethanol for 15-30 minutes.

5. Change gradually to water. (Pour out 1/3, add water, let sit 5 minutes, repeat 3 times).

6. Equilibrate in water for 5 minutes.

7. Stain in Carmine alum stain (section II.A.2. above) for 12-24 hours, depending on thickness (longer does not hurt it, but be consistent for all tissues of same age). The stain is reusable.

8. Soak in water for 30 seconds.

9. Soak in 70% ethanol for 15-30 minutes.

10. Soak in 95% ethanol for 15-30 minutes.

11. Soak in 100% ethanol for 20-30 minutes.

12. Clear in xylene for 4 to 72 hours, depending on the thickness of gland. The gland should be translucent after clearing. If any opaque (whitish) areas remain, place those slides in larger containers with xylene until translucent (clear).

13. Pipet enough Permount onto the tissue to cover the specimen and place a cover slip on top, being careful to avoid air bubbles. If bubbles form, lift the cover slip, pop the bubbles with the edge of a slide, and reapply the cover slip (additional Permount may be necessary).

C. **Helpful Hints**

In the early stages of this procedure, be especially careful to:

1. Allow adequate time for the mammary tissue to flatten. It must stick to the slide when the Parafilm is removed. If it doesn't, it will have to be reflattened, as the slides will be on their side in the staining dish.

2. Never pour washes or fixative directly onto the mammary tissue or it may come off the slide. In this event, the gland cannot be reattached. Slides should soak in the solutions, without further agitation.

3. Do not proceed to staining if the gland is not properly fixed in its center.

4. Examples of microscopic anatomy of the mammary gland are in Figures 2-4.

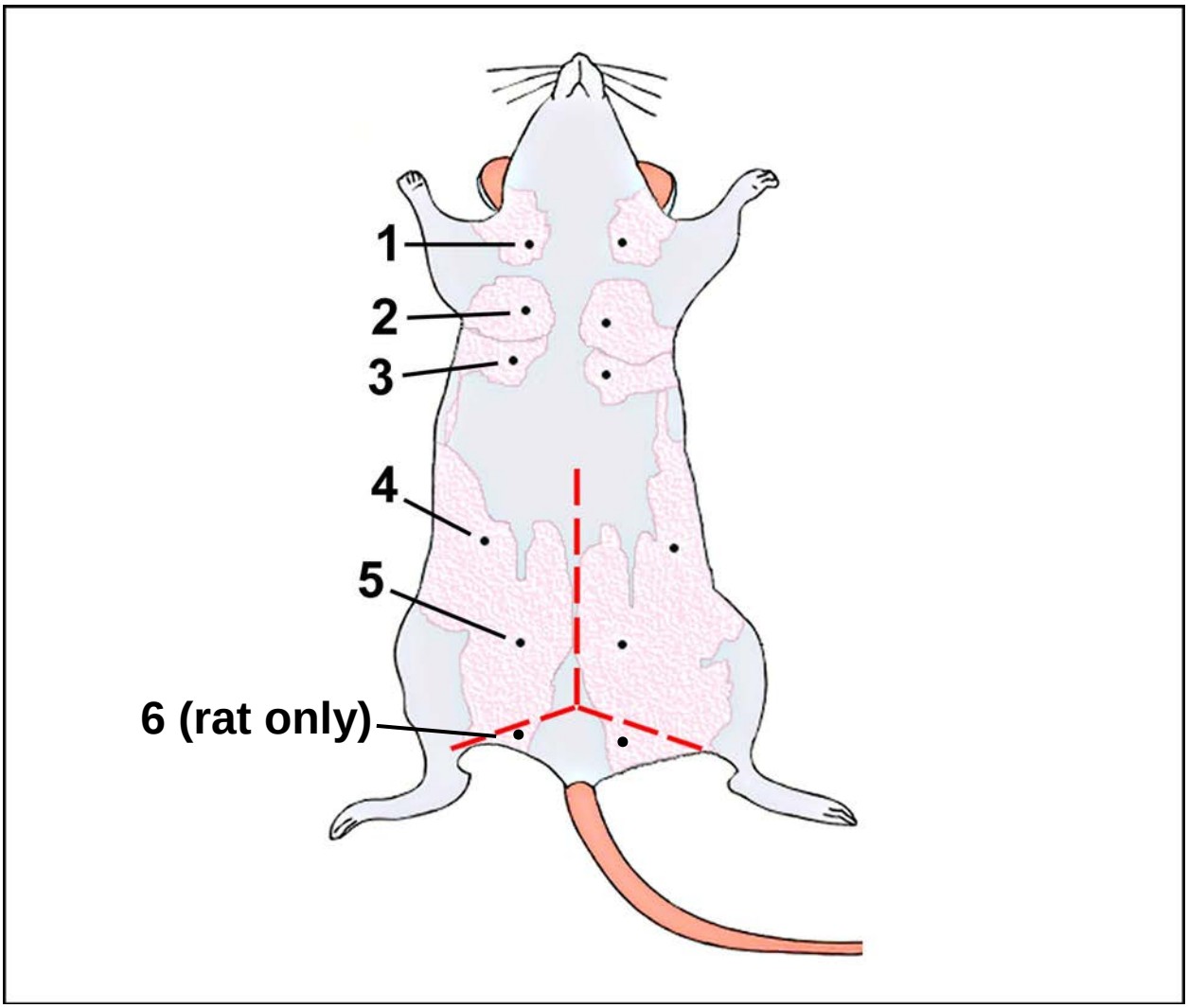


Figure 1. Schematic drawing of a mouse showing the 5 mammary glands and the locations of the nipples. In rats, there is a 6th mammary gland and nipple caudal to the 5th. The dashed line indicates the inverted-Y incision line for removal of the mammary gland fat pad. In the actual procedure, the limbs will be stretched out and down, and the incisions will extend farther down the medial aspect of the hind limbs.

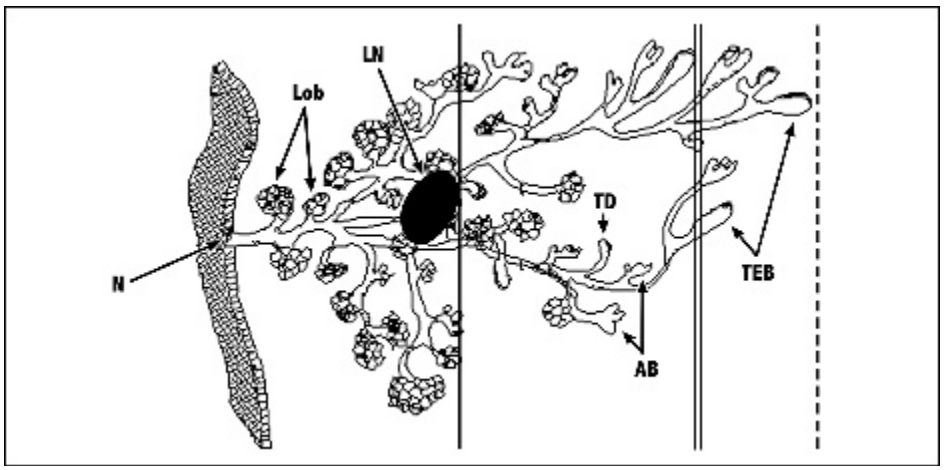


Figure 2. Schematic drawing of the microscopic anatomy of the rat mammary gland. The thick, solid line indicates the edge of the lymph node away from the nipple and the dotted line indicates the leading edge of gland growth (i.e., the most distal point of longitudinal growth. The arrow along the top of the figure indicates the direction of longitudinal growth. Longitudinal growth in an adult rat is measured from the thick, solid line to the dotted line. The thin double lines indicate the area two-thirds of the distance from the lymph node to the leading edge of gland growth where the degree of lateral branching is to be quantified, though the thin double lines would follow the curve of the leading edge of growth which cannot be depicted here. N – nipple, Lob – lobule, LN – lymph node, TD – terminal duct, AB – alveolar bud, TEB – terminal end bud.

From: Russo and Russo. (1996). *Environ Health Perspect* 104:938-67.

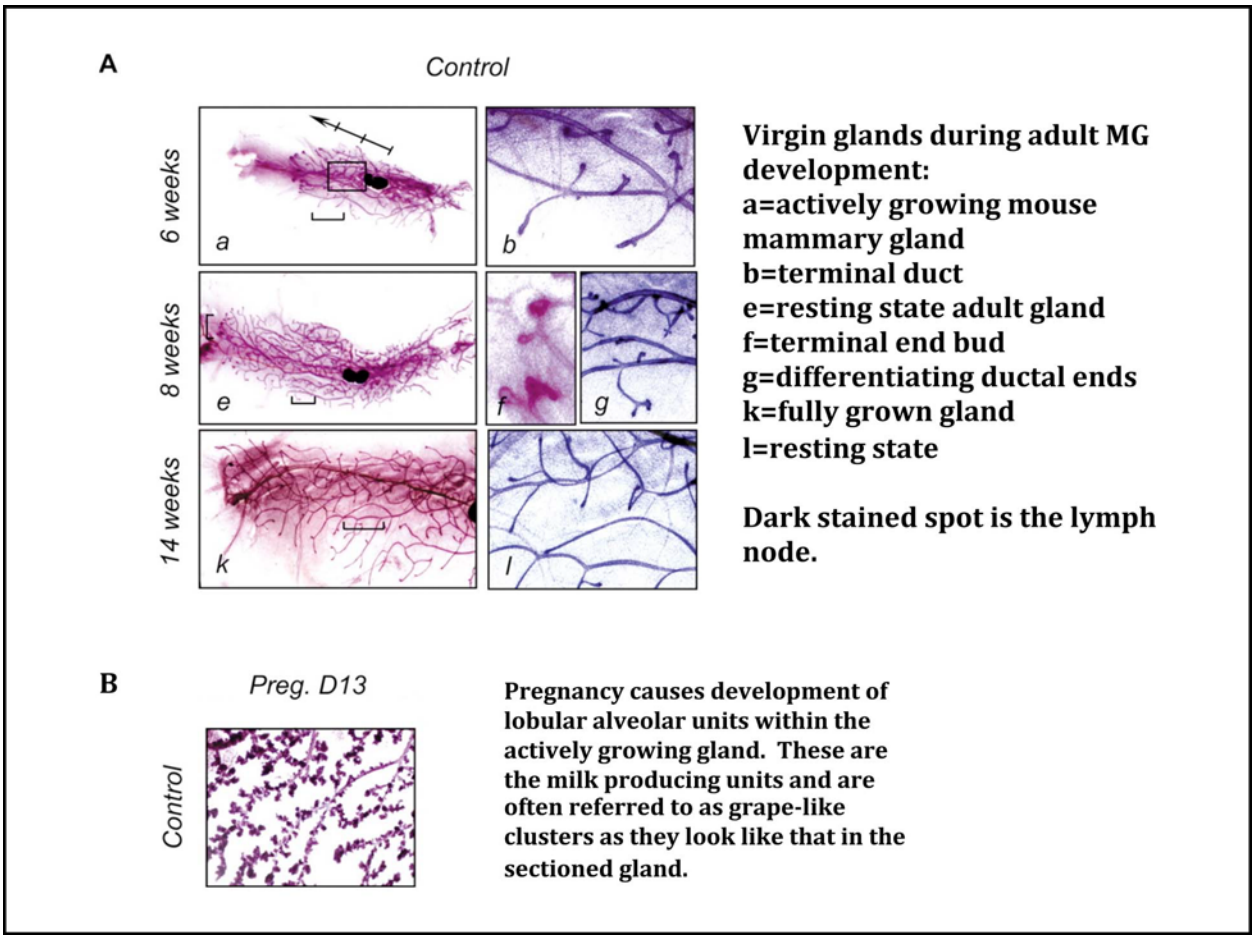


Figure 3. Control Mammary Gland Whole Mounts – Mouse.

From: Li, G et al. (2002). *Development* 129:4159-70

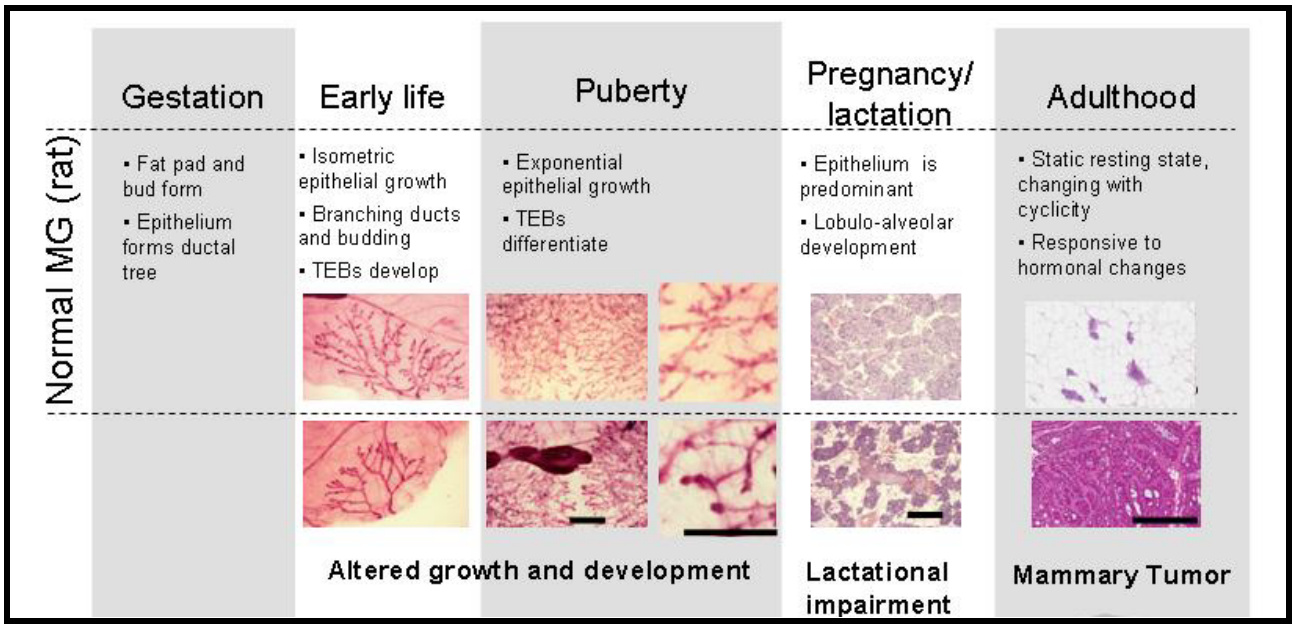


Figure 4: Stages of Mammary Gland development in the rat; normal and altered. Adapted from Rudel et al., 2011.

III. REFERENCES

1. NIH Guide to the Analysis of the Mammary Gland http://mammary.nih.gov/methodcd/methodcd.html
2. Development of the Mammary Gland: A Whole Mount Analysis http://mammary.nih.gov/atlas/wholemounts/normal/index.html
3. Li G, Robinson GW, Lesche R, Martinez-Diaz H, Jiang Z, Rozengurt N, Wagner K-U, Wu D-C, Lane TF, Liu X, Hennighausen L, Wu H (2002). Conditional loss of PTEN leads to precocious development and neoplasia in the mammary gland. *Development* 129 (17):4159-4170.
4. Russo IH, Russo J. (1996). Mammary gland neoplasia in long-term rodent studies. *Environ Health Perspect*. 104(9):938-967.
5. S.E. Fenton. 2006. Endocrine Disrupting Compounds and Mammary Gland Development: Early exposure and later life consequences. *Endocrinology* 147(Supplement):S18-S24.
6. Fenton, S.E., J.T. Hamm, L.S. Birnbaum, and G.L. Youngblood. 2002. Early adverse effects of prenatal exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in the rat mammary gland *Toxicol. Sci.* 67: 63-74.
7. Rudel, R.A., Fenton, S.E., Ackerman, J.M., Euling, S.Y., Makris, S.L. Environmental Exposures and Mammary Gland Development: State of the Science, Public Health Implications, and Research Recommendations. *Environ Health Perspect*, In press.

APPENDIX 7 - General Information for Neurobehavioral Studies

In some studies, the protocol may require evaluation of animals in a battery of tests to provide a functional evaluation of the development of the nervous system. Animals shall be evaluated for motor, sensory and cognitive function with specific targeted tests at different ages. There shall typically be 10-20 animals/sex/group in these tests. Histological evaluation of nervous system development shall be conducted if required by the protocol.

Post-weaning testing shall occur in a defined dedicated area outside of the animal housing room. To control for exposure group and testing device bias, animals shall be counter-balanced across testing devices for each test session. Testing devices shall be wiped clean with an appropriate decontaminant between animals to reduce possible influence of odors.

Motor Function: Motor activity shall be assessed by an automated activity recording apparatus at ages indicated in the test-article-specific protocol. The testing system shall elicit exploratory behavior from the test animal so that habituation to its environment can be demonstrated. The session length shall be long enough to show habituation by the last 20% of the test session for the control animals, and all sessions shall be of the same duration for each age group (e.g., one-hour for adults and 30 minutes for young animals). Locomotor activity shall be recorded in equal 5 minute epochs over the session. Assessment shall be conducted by either an automated photocell device with appropriate sensitivity and spacing of photocells to accommodate the young as well as adult animals, or alternatively a video tracking system for assessment of total and patterned activity. Gait analysis may also be conducted to evaluate motor function of the animals.

Sensory Evaluation: Auditory startle/prepulse-startle inhibition test: This test shall generally be performed in young adult animals, at ages indicated in the test-article-specific protocol. A computer assisted automated system shall be used that has been demonstrated to show startle, startle inhibition and habituation. Individual devices shall be contained in sound-attenuated chambers. Each system shall be appropriately calibrated before the start of each study, and on a weekly basis during the conduct of a study.

Learning and Memory: A test of learning and memory shall be conducted in young adult animals at ages indicated by the test article-specific protocol. The testing paradigm chosen shall depend on the test article. Acceptable testing paradigms include, but are not limited to, performance in various mazes and running wheel, as well as active and passive avoidance, social behavior and fear conditioning. Specific details for these tests will be included in test-article-specific protocols if required.

Histological Evaluation: Histological evaluation of animals at various ages may be required in some studies. Details of such evaluations are test article specific and will be included in the NTP test-article-specific protocol. For some studies, specified brain areas shall be collected, and frozen for shipment to an NTP-specified laboratory for molecular or other analysis.

Specific details for neurobehavioral testing will be included in test article-specific protocols requiring these analyses.